

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

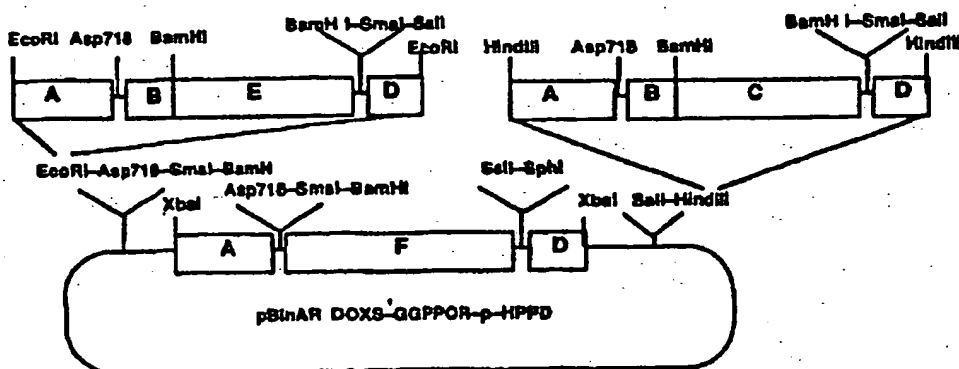
**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



- (72) REINDL, ANDREAS, DE  
(72) LEON MEJIA, PATRICIA, MX  
(72) ESTEVES PALMAS, JUAN MANUEL, MX  
(72) CANTERO GRACIA, MARIA ARACELI, MX  
(72) EBNETH, MARCUS, DE  
(72) HERBERS, KARIN, DE  
(71) SUNGENE GMBH & CO.KGAA, DE  
(51) Int.Cl.<sup>7</sup> C12N 15/53, C12N 15/82, C12N 15/54, C12N 9/10,  
C12N 9/04, C12Q 1/02, A01H 5/00  
(30) 1998/08/05 (198 35 219.0) DE  
(30) 1998/10/01 (198 45 216.0) DE  
(30) 1998/10/01 (198 45 224.1) DE  
(30) 1998/10/01 (198 45 231.4) DE  
(54) SEQUENCE ADN CODANT POUR UNE 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE ET SA SURPRODUCTION DANS LES PLANTES  
(54) DNA SEQUENCE CODING FOR A 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE AND THE OVERPRODUCTION THEREOF IN PLANTS

BINARY VECTOR FOR OVEREXPRESSING THE DOXS-GENE FROM E. COLI, THE GGPPOR GENE FROM ARABIDOPSIS THALIANA AND THE HPPD GENE FROM STREPTOMYCES AVERMITILIS IN THE PLASTIDS OF TRANSGENIC PLANTS



- (57) Method for the production of plants with enhanced vitamin E biosynthesis efficiency by overproduction of a 1-deoxy-D-xylulose-5-phosphate synthase gene from Arabidopsis or E. coli.



**PCT**  
WELTORGANISATION FÜR GEISTIGES EIGENTUM  
Internationales Büro  
INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE  
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

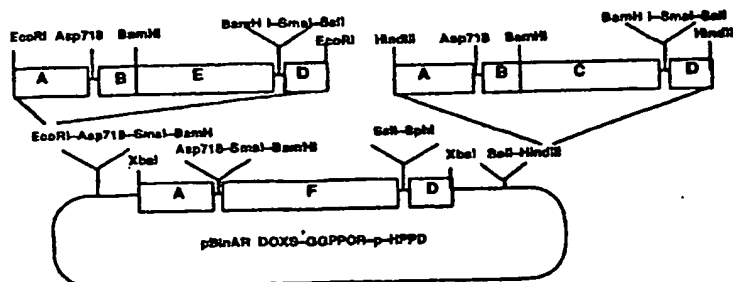
(51) Internationale Patentklassifikation <sup>7</sup> : C12N 15/53, 15/54, 15/82, 9/10, 9/04, C12Q 1/02, A01H 5/00		A1	(11) Internationale Veröffentlichungsnummer: WO 00/08169
		(43) Internationales Veröffentlichungsdatum:	17. Februar 2000 (17.02.00)
(21) Internationales Aktenzeichen: PCT/EP99/05467		(74) Anwalt: LANGFINGER, Klaus-Dieter, BASF Aktiengesellschaft, D-67056 Ludwigshafen (DE).	
(22) Internationales Anmeldedatum: 30. Juli 1999 (30.07.99)		(81) Bestimmungsstaaten: AL, AU, BG, BR, BY, CA, CN, CZ, GE, HR, HU, ID, IL, IN, JP, KR, KZ, LT, LV, MK, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TR, UA, US, ZA, eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Prioritätsdaten:			
198 35 219.0	5. August 1998 (05.08.98)	DE	
198 45 216.0	1. Oktober 1998 (01.10.98)	DE	
198 45 231.4	1. Oktober 1998 (01.10.98)	DE	
198 45 224.1	1. Oktober 1998 (01.10.98)	DE	
(71) Anmelder (für alle Bestimmungsstaaten ausser US): SUN-GENE GMBH & CO. KGAA [DE/DE]; Corrensstrasse 3, D-06468 Gatersleben (DE).		Veröffentlicht Mit internationalem Recherchenbericht. Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.	
(72) Erfinder; und (75) Erfinder/Anmelder (nur für US): REINDL, Andreas [DE/DE]; Albertine-Scherer-Strasse 21, D-67134 Birkenheide (DE). MEJIA, Patricia Leon [MX/MX]; Ganizalo de Sandoval 226, Cuernavaca, Morelos 62250 (MX). PALMAS, Juan Manuel Esteves [MX/MX]; Entrada a Ojo de Agua Col., Loma Bonita Tecamac Estado (MX). GRACIA, Maria Araceli Canter [MX/MX]; 2da Privad Los Pinos 22, Loma Bonita Cuernavaca, Morelos 62210 (MX). EBNETH, Marcus [DE/DE]; Bicklingerweg 16, D-06486 Quedlinburg (DE). HERBERS, Karin [DE/DE]; Am Hange 6, D-06484 Quedlinburg (DE).		814/00006 030501	

(54) Title: DNA SEQUENCE CODING FOR A 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE AND THE OVERPRODUCTION THEREOF IN PLANTS

(54) Bezeichnung: DNA-SEQUENZ KODIEREND FÜR EINE 1-DEOXY-D-XYLULOSE-5-PHOSPHAT SYNTHASE UND DEREN ÜBERPRODUKTION IN PFLANZEN

Binärer Vektor zur Überexpression des DOXS-Gens aus *E. coli*, des GGPPOR-Gens aus *Arabidopsis thaliana* und des HPPD-Gens aus *Streptomyces avermitilis* in den Plastiden transgener Pflanzen.

BINARY VECTOR FOR OVEREXPRESSION THE DOXS-GENE FROM *E. COLI*, THE GGPPOR GENE FROM *ARABIDOPSIS THALIANA* AND THE HPPD GENE FROM *STREPTOMYCES AVERMITILIS* IN THE PLASTIDS OF TRANSGENIC PLANTS



(57) Abstract

Method for the production of plants with enhanced vitamin E biosynthesis efficiency by overproduction of a 1-deoxy-D-xylulose-5-phosphate synthase gene from *Arabidopsis* or *E. coli*.

Mec

DNA SEQUENCE CODING FOR A 1-DEOXY-D-XYLULOSE-5-PHOSPHATE  
SYNTHASE AND THE OVERPRODUCTION THEREOF IN PLANTS

The present invention relates to the use of DNA sequences coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, specifically to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or of a DNA sequence hybridizing with the latter, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of a DNA sequence SEQ ID No. 5 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a p-dihydroxyphenylpyruvate dioxygenase (HPPD) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3, of a DNA sequence SEQ ID No. 5 and of a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS), a hydroxyphenylpyruvate dioxygenase (HPPD) and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to processes for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, comprising a DNA sequence SEQ-ID No. 1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, to the plants themselves produced in this way, and to the use of SEQ ID No. 1 or SEQ ID No. 3 for producing a test system for identifying DOXS inhibitors.

An important aim of molecular genetic work on plants to date has been the generation of plants with increased content of sugars, enzymes and amino acids. However, there is also commercial interest in the development of plants with increased content of vitamins, such as increasing the tocopherol content.

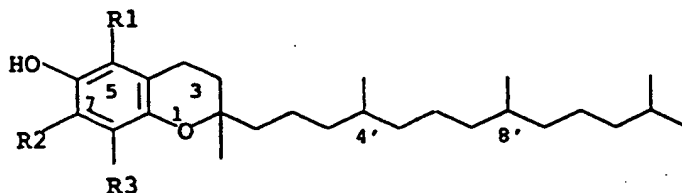
The eight compounds with vitamin E activity which occur in nature are derivatives of 6-chromanol (Ullmann's Encyclopedia of Industrial Chemistry, Vol. A 27 (1996), VCH Verlagsgesellschaft,

0817/00006

## 2

Chapter 4, 478-488, Vitamin E). The first group (1a-d) is derived from tocopherol, while the second group consists of derivatives of tocotrienol (2a- d):

5



10

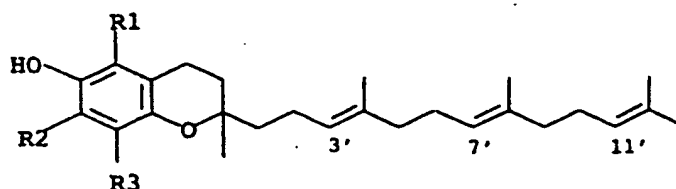
1a,  $\alpha$ -Tocopherol:  $R^1 = R^2 = R^3 = \text{CH}_3$

1b,  $\beta$ -Tocopherol [148-03-8]:  $R^1 = R^3 = \text{CH}_3$ ,  $R^2 = \text{H}$

15 1c,  $\gamma$ -Tocopherol [54-28-4]:  $R^1 = \text{H}$ ,  $R^2 = R^3 = \text{CH}_3$

1d,  $\delta$ -Tocopherol [119-13-1]:  $R^1 = R^2 = \text{H}$ ,  $R^3 = \text{CH}_3$

20



25

2a,  $\alpha$ -Tocotrienol [1721-51-3]:  $R^1 = R^2 = R^3 = \text{CH}_3$

2b,  $\beta$ -Tocotrienol [490-23-3]:  $R^1 = R^3 = \text{CH}_3$ ,  $R^2 = \text{H}$

2c,  $\gamma$ -Tocotrienol [14101-61-2]:  $R^1 = \text{H}$ ,  $R^2 = R^3 = \text{CH}_3$

2d,  $\delta$ -Tocotrienol [25612-59-3]:  $R^1 = R^2 = \text{H}$ ,  $R^3 = \text{CH}_3$

30

The compound of great commercial importance is  $\alpha$ -tocopherol.

There are limits on the development of crop plants with increased tocopherol content through tissue culture or seed mutagenesis and natural selection. Thus, on the one hand, the tocopherol content must be measurable even in the tissue culture and, on the other hand, the only plants which can be manipulated by tissue culture techniques are those which can be regenerated to whole plants from cell cultures. In addition, crop plants may, after mutagenesis and selection, show unwanted properties which must be eliminated again by backcrossings, several times in some instances. Moreover increasing the tocopherol content by crossing would be restricted to plants of the same species.

45 For these reasons, the genetic engineering procedure of isolating, and transferring into target crop plants, an essential biosynthesis gene coding for tocopherol synthesis activity is

0817/00006

3

superior to the classical breeding method. The preconditions for this process are that the biosynthesis and its regulation are known and that genes which influence the biosynthetic activity are identified.

5

Isoprenoids or terpenoids consist of various classes of lipid-soluble molecules and are formed partly or completely of  $C_5$ -isoprene units. Pure prenyl lipids (e.g. carotenoids) consist of C skeletons derived exclusively from isoprene units, whereas  
10 mixed prenyl lipids (e.g. chlorophyll) have an isoprenoid side chain connected to an aromatic nucleus.

The biosynthesis of prenyl lipids starts from 3 x acetyl-CoA units, which are converted via  $\beta$ -hydroxymethylglutaryl-CoA  
15 (HMG-CoA) and mevalonate into the initial isoprene unit ( $C_5$ ), isopentenyl pyrophosphate (IPP). It has recently been shown by in vivo feeding experiments with  $C^{13}$  that a mevalonate-independent pathway is followed in various eubacteria, green algae and plant chloroplasts to produce IPP:

20

25

30

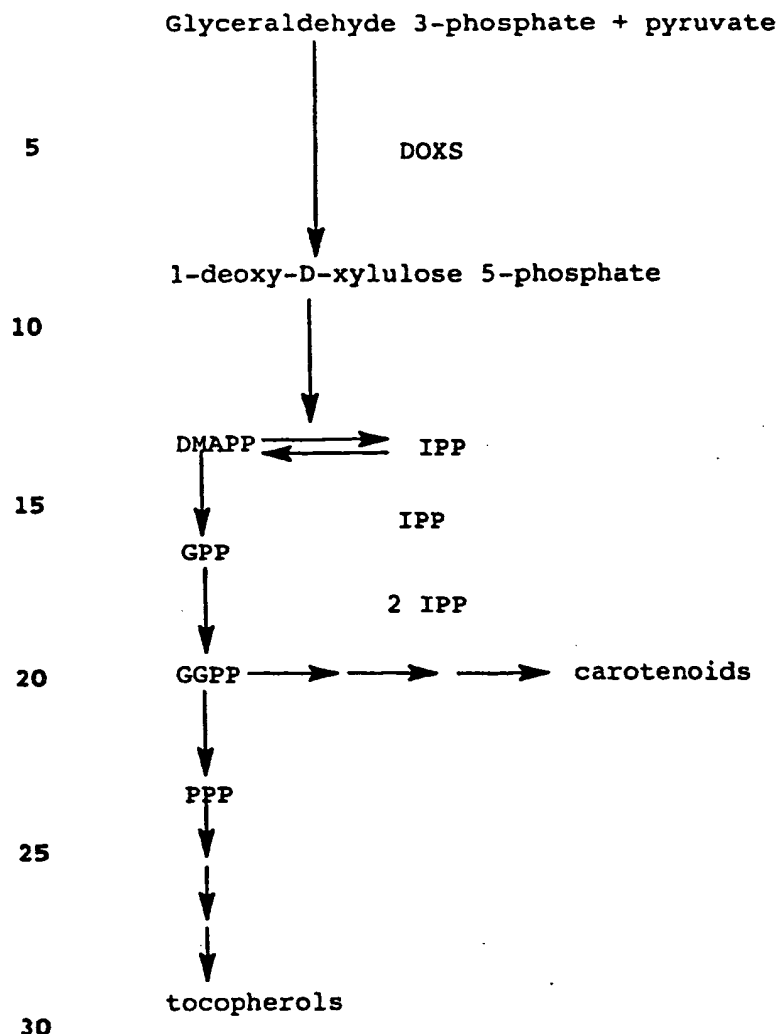
35

40

45

0817/00006

4



This entails hydroxyethylthiamine, which is produced by decarboxylation of pyruvate, and glyceraldehyde 3-phosphate (3-GAP) being converted, in a "transketolase" reaction mediated by 1-deoxy-D-xylulose-5-phosphate synthase, initially into 1-deoxy-D-xylulose-5-phosphate (Schwender et al., FEBS Lett. 414(1), 129-134(1997); Arigoni et al., Proc.Natl.Acad.Sci USA 94(2), 10600-10605 (1997); Lange et al., Proc.Natl.Acad.Sci.USA 95(5), 2100-2104(1998); Lichtenthaler et al., FEBS Lett. 400(3), 271-274(1997). The latter is then converted by an intramolecular rearrangement into IPP (Arigoni et al., 1997). Biochemical data indicate that the mevalonate pathway operates in the cytosol and leads to the production of phytosterols. The antibiotic mevinolin, a specific inhibitor of mevalonate production, leads only to inhibition of sterol biosynthesis in the cytoplasm, whereas prenyl lipid production in the plastids is unaffected (Bach and Lichtenthaler, Physiol. Plant 59(1983), 50-60. By

0817/00006

## 5

contrast, the mevalonate-independent pathway has a plastidic localization and leads mainly to the production of carotenoids and plastidic prenyl lipids (Schwender et al., 1997; Arigoni et al., 1997).

5

IPP is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMAPP). Condensation of IPP with DMAPP in a head-tail addition affords the monoterpene (C<sub>10</sub>) geranyl pyrophosphate (GPP). Addition of further IPP units results in the sesquiterpene

- 10 (C<sub>15</sub>) farnesyl pyrophosphate (FPP) and the diterpene (C<sub>20</sub>) geranyl-geranyl pyrophosphate (GGPP). Linkage of two GGPP molecules results in the production of the C<sub>40</sub> precursors for carotenoids. GGPP is transformed by a prenyl chain hydrogenase into phytyl pyrophosphate (PPP), the starting material for
- 15 further production of tocopherols.

The ring structures of the mixed prenyl lipids which lead to the production of vitamins E and K comprise quinones whose initial metabolites are derived from the shikimate pathway. The aromatic

20 amino acids phenylalanine and tyrosine are converted into hydroxyphenylpyruvate, which is transformed by dioxygenation into homogentisic acid. The latter is bound to PPP in order to produce 2-methyl-6-phytylquinol, the precursor of  $\alpha$ -tocopherol and  $\alpha$ -tocoquinone. Methylation steps with S-adenosylmethionine as

25 methyl group donor result initially in 2,3-dimethyl-6-phytylquinol and then, by cyclization, in  $\gamma$ -tocopherol and, by methylation again, in  $\alpha$ -tocopherol (Richter, Biochemie der Pflanzen, Georg Thieme Verlag Stuttgart, 1996).

- 30 Examples are to be found in the literature showing that manipulation of an enzyme can influence the direction of the metabolite flux. It was possible in experiments with modified expression of phytoene synthase, which links two GGPP molecules together to give 15-cis-phytoene, to measure a direct effect on
- 35 the amounts of carotenoids in these transgenic tomato plants (Fray and Grierson, Plant Mol. Biol. 22(4), 589-602(1993); Fray et al., Plant J., 8, 693-701(1995). As expected, transgenic tobacco plants with reduced amounts of phenylalanine-ammonium lyase show reduced phenylpropanoid amounts. The enzyme
- 40 phenylalanine-ammonium lyase catalyzes the breakdown of phenylalanine and thus removes it from phenylpropanoid biosynthesis (Bate et al., Proc. Natl. Acad. Sci USA 91 (16): 7608-7612 (1994); Howles et al., Plant Physiol. 112. 1617-1624(1996).

45



0817/00006

## 6

To date, little has been disclosed about increasing the metabolite flux in order to increase the tocopherol content of plants through overexpression of individual biosynthesis genes. There is merely a description in WO 97/27285 of modification of the tocopherol content by increased expression or by down-regulation of the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD).

It is an object of the present invention to develop a transgenic plant with increased content of tocopherols, vitamin K, chlorophylls and carotenoids.

We have found that this object is achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate sythase (DOXS) gene in the plants.

15

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity in plants was increased by overexpression of the homologous gene (gene from organism of the same species). This can also be achieved by expressing a heterologous gene (gene from remote organisms). Nucleotide sequences from *Arabidopsis thaliana* DOXS (Acc. No. U 27099), rice (Acc. No. AF024512) and peppermint (Acc. No. AF019383) are described.

In one example 1 there is enhanced expression of the DOXS gene from *Arabidopsis thaliana* (SEQ ID No.:1; Mandel et al., Plant J. 9, 649-658(1996); Acc. No. U27099) in transgenic plants. Plastidic localization is ensured by the transit signal sequence present in the gene sequence. A suitable expression cassette is also a DNA sequence which codes for a DOXS gene which hybridizes with SEQ ID No. 1 and which is derived from other organisms such as, for example, *E. coli* (SEQ ID No.3) or, preferably, from other plants.

The GGPP which is now available in increased quantities is converted further in the direction of tocopherols and carotenoids.

40

Efficient production of carotenoids is essential for photosynthesis, where they serve together with chlorophylls as "light-collecting complexes" for better utilization of the energy of photons (Heldt, *Pflanzenbiochemie. Spektrum Akademischer Verlag Heidelberg Berlin Oxford*, 1996). In addition, carotenoids carry out important functions protecting from oxygen free radicals such as singlet oxygen, which they are able to return to

0817/00006

7

the ground state (Asada, 1994; Demming-Adams and Adams, Trends in Plant Sciences 1; 21-26(1996). A 1-deoxy-D-xylulose-5-phosphate synthase-defective *Arabidopsis thaliana* mutant showing an "albino phenotype" has been isolated (Mandel et al., 1996). It can be  
 5 inferred from this that a reduced amount of carotenoids in the plastids has adverse effects on the plant.

We have found that the object is also achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene and of a  
 10 p-hydroxyphenylpyruvate dioxygenase (HPPD) gene in the plants, see Figure 1.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general  
 15 starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity in transgenic tobacco and oilseed rape plants was increased by overexpression of the DOXS from *E. coli*. This can be achieved by expression of homologous or other heterologous genes.

20 The D-1-deoxy-xylulose 5-phosphate which is now available in increased quantities is converted further in the direction of tocopherols and carotenoids.

25 In addition, the production of homogentisic acid further intensifies the metabolite flux in the direction of phytylquinones and thus tocopherol, see Figure 1. Homogentisic acid is produced from p-hydroxyphenylpyruvate by the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD). cDNAs coding for this  
 30 enzyme have been described from various organisms such as, for example, from microorganisms, from plants and from humans.

In Example 11 there was for the first time overexpression of the HPPD gene from *Streptomyces avermitilis* (Denoya et al.,  
 35 J. Bacteriol. 176(1994), 5312-5319; SEQ ID No. 5) together with the DOXS from *E. coli* SEQ ID No. 3 in plants and plant plastids.

The increase in the plastidic IPP production leads to enhanced production of all plastidic isoprenoids. The increased provision  
 40 of homogentisic acid ensures that sufficient substrate is available for the production of tocopherols in the plastids. This homogentisate which is now available in increased quantities can in turn be converted in the transgenic plants with the amount, which is increased due to the overexpression of DOXS, of phytyl  
 45 diphosphate (PPP). PPP occupies a key position, in this connection, because it serves on the one hand as starting

0817/00006

8

substrate for chlorophylls and phylloquinones, and on the other hand for tocopherols.

The transgenic plants are produced by transformation of the  
5 plants with a construct containing the DOXS and HPPD genes. Tobacco and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and carotenoids.

- 10 The invention also relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5, which code for a DOXS or HPPD or functional equivalents thereof, for producing a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these  
15 cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS or HPPD and conferring on the host the ability to overproduce tocopherol.
- 20 The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a  
25 polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS or HPPD gene located in between.

- An expression cassette is produced by fusing a suitable promoter  
30 to a suitable DOXS or HPPD DNA sequence and preferably a DNA which is inserted between promoter and DOXS or HPPD DNA sequence and codes for a chloroplast-specific transit peptide, and a polyadenylation signal by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F.  
35 Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in  
40 Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

- It is also possible to use expression cassettes whose DNA sequence codes for a DOXS or HPPD fusion protein, where part of  
45 the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides which are specific for chloroplasts and which are eliminated enzymatically

0817/00006

9

from the DOXS or HPPD part after translocation of the DOXS or HPPD gene into the chloroplasts are preferred. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (for example the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene and an HPPD gene is preferably cloned into a vector, for example pBin19, which is suitable for transformation of *Agrobacterium tumefaciens*.

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

20

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

The invention further relates to:

- 40 - Process for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and a DNA sequence SEQ ID No. 5 or DNA sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,

45

0817/00006

## 10

- Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS and an HPPD DNA sequence in plants.

The object have also been achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene and of a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) gene in the 10 plants, see Figure 1.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. 15 For this purpose, the DOXS activity in transgenic tobacco and oilseed rape plants was increased by overexpression of the DOXS from *E. coli*. This can be achieved by expression of homologous or other heterologous genes.

20 In order to convert the GGPP which is now available in increased quantities in the direction of tocopherols and carotenoids, in a further step essential to the invention in addition the activity of the enzyme geranylgeranyl-pyrophosphate oxidoreductase is increased by overexpression of a corresponding gene. This measure 25 achieves an increased production of phytyl pyrophosphate through increased conversion of geranylgeranyl pyrophosphate into phytyl pyrophosphate.

This is done, for example, by enhanced expression of the GGPPOR 30 gene from *Arabidopsis thaliana* (SEQ ID No. 7) in transgenic plants. In order to ensure plastidic localization, a transit signal sequence is put in front of the *Arabidopsis* GGPPOR. Also suitable as expression cassette is a DNA sequence coding for a GGPPOR gene which hybridizes with SEQ ID No. 7 and which is 35 derived from other organisms or from other plants.

Example 15 describes the cloning of the GGPPOR gene from *Arabidopsis thaliana*.

40 Increasing the plastidic 1-deoxy-D-xylulose 5-phosphate and phytyl pyrophosphate production leads to increased production of all plastidic isoprenoids, so that sufficient substrate for the production of tocopherols, chlorophylls, vitamin K and phylloquinones is available in the plastids.

45

0817/00006

## 11

The transgenic plants are produced by transformation of the plants with a construct containing the DOXS and GGPPOR genes. Tobacco and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and 5 carotenoids.

The invention also relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7, which code for a DOXS or GGPPOR or functional equivalents thereof, for producing plants 10 with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS or GGPPOR and conferring on the 15 host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an 20 expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS or GGPPOR gene located in between. Operative linkage 25 means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but 30 are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from 35 tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. 1 shows 40 the tobacco transformation vectors pBinAR-Hyg with the 35S promoter (A) and pBinAR-Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- 45 - OCS: octopine synthase terminator
- PNOS: nopaline synthase promoter

0817/00006

## 12

- also drawn in are those restriction cleavage sites which cut the vector only once.

Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS or GGPPOR gene in the plant can be controlled at a particular time. Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP-A 388186), a tetracycline-inducible (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible (EP-A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

25

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445 - 245).

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS or GGPPOR DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS or GGPPOR DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

0817/00006

## 13

It is also possible to use expression cassettes whose DNA sequence codes for a DOXS or GGPPOR fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS or GGPPOR part after translocation of the DOXS or GGPPOR gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene or a GGPPOR gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming *Agrobacterium tumefaciens*.

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

The invention further relates to:

- Process for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or a DNA sequence SEQ ID No. 3 and a SEQ ID No. 7 or DNA



0817/00006

## 14

sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,

- Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS and a GGPPOR DNA sequence in plants.
- 10 The object have also been achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene, a p-hydroxyphenylpyruvate dioxygenase (HPPD) gene and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) gene in the plants, see Figure 1.
- 15 In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity was increased by
- 20 overexpression of the DOXS from E. coli in transgenic tobacco and oilseed rape plants. This can also be achieved by expressing homologous or other heterologous DOXS genes - such as, for example, a DNA sequence SEQ ID No. 1.
- 25 The D-1-deoxy-xylulose 5-phosphate which is now available in increased quantities is converted further in the direction of geranylgeranyl pyrophosphate.

In order to convert the GGPP which is now available in increased quantities in the direction of tocopherols and carotenoids, in a further step essential to the invention in addition the activity of the enzyme geranylgeranyl pyrophosphate oxidoreductase is increased by overexpression of a corresponding homologous or heterologous gene. This measure achieves an increased production

35 of phytyl pyrophosphate through increased conversion of geranylgeranyl pyrophosphate into phytyl pyrophosphate.

This done, for example, by enhanced expression of the GGPPOR gene from Arabidopsis thaliana (SEQ ID No. 7) in transgenic plants. In

40 order to ensure plastidic localization, a transit signal sequence is put in front of the Arabidopsis GGPPOR. Also suitable as expression cassette is a DNA sequence coding for a GGPPOR gene which hybridizes with SEQ ID No. 7 and which is derived from other organisms or from other plants.

0817/00006

## 15

Example 15 describes the cloning of the GGPPOR gene from *Arabidopsis thaliana*.

In order to convert the PPP which is now available in increased quantities in the direction of tocopherol and carotenoids, in a further step essential to the invention in addition the activity of the enzyme p-hydroxylphenylpyruvate dioxygenase (HPPD) is increased by overexpression of a corresponding homologous or heterologous gene. This measure achieves increased production of homogentisic acid by increased conversion of hydroxyphenylpyruvate into homogentisic acid.

cDNAs coding for this enzyme have been described from various organisms such as, for example, from microorganisms, from plants and from humans.

Example 10 describes the cloning of the HPPD gene from *Streptomyces avermitilis* (Denoya et al., J. Bacteriol. 176(1994), 5312-5319; SEQ ID No. 5). In order to ensure a plastidic localization, a transit signal sequence is put in front of the *Streptomyces* HPPD. Also suitable as expression cassette is a DNA sequence which codes for an HPPD gene which hybridizes with SEQ ID No. 5 and is derived from other organisms or from plants.

The increase in the plastidic D-1-deoxy-xylulose 5-phosphate, the phytyl pyrophosphate and the homogentisic acid production leads to increased production of all plastidic isoprenoids. The increased provision of these precursors ensures that sufficient substrate is available for the production of tocopherols, chlorophylls, vitamin K and phylloquinones in the plastids.

The transgenic plants according to the invention are produced by transforming the plants with a construct containing the DOXS, the HPPD gene and the GGPPOR gene (Example 17). Tobacco and oilseed rape were employed as model plants for producing tocopherols, vitamin K, chlorophylls and carotenoids.

The invention relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ-ID No. 7, which code for a DOXS, an HPPD and a GGPPOR or functional equivalents thereof to produce a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS, an HPPD and a GGPPOR and conferring on the host the ability to overproduce tocopherol.

0817/00006

## 16

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS, the HPPD or the GGPPOR gene located in between. Operative linkage means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. 2 shows the tobacco transformation vectors pBinAR-Hyg with the 35S promoter (A) and pBinAR-Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- OCS: octopine synthase terminator
- 30 - PNOS: nopaline synthase promoter
- also drawn in are those restriction cleavage sites which cut the vector only once.

Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

45 The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS, HPPD and GGPPOR gene in the plant can be controlled at a particular time.

0817/00006

## 17

Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP-A 388186), a tetracycline-inducible (Gatz et al., (1992) 5 Plant J. 2, 397-404), an abscisic acid-inducible (EP-A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

Further particularly preferred promoters are those which ensure 10 expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus 15 et al., EMBO J. 8 (1989) 2445 - 245).

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS, HPPD and GGPPOR DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS, HPPD and 20 GGPPOR DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring 25 Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

30 It is also possible to use expression cassettes whose DNA sequence codes for a DOXS, HPPD and GGPPOR fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for 35 chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS, HPPD and GGPPOR part after translocation of the DOXS, HPPD and GGPPOR gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional 40 equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene, an HPPD gene or a GGPPOR gene is preferably cloned into a vector, for 45 example pBin19, which is suitable for transforming *Agrobacterium tumefaciens*.

0817/00006

18

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

- 15 The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants.
- 20 Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

25

The invention further relates to:

- Processes for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3, a DNA sequence SEQ ID No. 5 and a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,
- 35 - Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS, an HPPD and an GGPPOR DNA sequence in plants.

40

It was therefore an additional object of the present invention to develop a test system for identifying DOXS inhibitors.

45

0817/00006

## 19

This object has been achieved by expressing a DOXS gene from Arabidopsis or E. coli, or DNA sequences hybridizing therewith, and subsequently testing chemicals for inhibition of the DOXS enzyme activity.

5

The transgenic plants are produced by transforming the plants with a construct containing the DOXS gene. Arabidopsis and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and carotenoids.

10

Cloning of the complete DOXS gene from Arabidopsis takes place by isolating the cDNA (SEQ ID No. 1) specific for the DOXS gene.

The invention relates to the use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 which codes for a DOXS or functional equivalent thereof for producing a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid content. The nucleic acid sequence can moreover be, for example, a DNA or cDNA sequence. Examples of coding sequences suitable for insertion into an expression cassette are those which code for a DOXS and which confer on the host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises a promoter upstream, i.e. at the 5' end of the coding sequence, and a polyadenylation signal downstream, i.e. at the 3' end, and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS gene located in between. Operative linkage means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987) 8693 - 8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. [lacuna] shows the tobacco transformation vectors pBinAR-Hyg with the 35S

0817/00006

## 20

promoter (A) and pBinAR-Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- 5 - OCS: octopine synthase terminator
- PNOS: nopaline synthase promoter
- also drawn in are those restriction cleavage sites which cut the vector only once.

10 Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

20

The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS gene in the plant can be controlled at a particular time. Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 25 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP-A 388186), a tetracycline-inducible (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible (EP-A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular 35 mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445 - 245).

40 It has been possible with the aid of a seed-specific promoter to express a foreign protein stably up to a content of 0.67% of the total soluble seed protein in the seeds of transgenic tobacco plants (Fiedler and Conrad, Bio/Technology 10 (1995), 1090-1094). The expression cassette can therefore contain, for example, a 45 seed-specific promoter (preferably the phaseolin promoter (US 5504200), the USP (Baumlein, H. et al. Mol. Gen. Genet. (1991) 225 (3), 459 - 467) or LEB4 promoter (Fiedler and Conrad,

0817/00006

21

1995)), the LEB4 signal peptide, the gene to be expressed, and an ER retention signal. The construction of a cassette of this type is depicted diagrammatically by way of example in Figure 2.

- 5 An expression cassette is produced by fusing a suitable promoter to a suitable DOXS DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience (1987).

- Particularly preferred sequences are those which ensure targeting in the apoplast, in plastids, in the vacuole, in the mitochondrion, in the endoplasmic reticulum (ER) or, due to absence of appropriate operative sequences, ensure retention in the compartment of production, the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285 - 423). Localization in the ER has proved particularly beneficial for the amount of protein accumulated in transgenic plants (Schouten et al., Plant Mol. Biol. 30 (1996), 781 - 792).

- It is also possible to use expression cassettes whose DNA sequence codes for a DOXS fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS part after translocation of the DOXS gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

- The inserted nucleotide sequence coding for a DOXS can be prepared by synthesis or be obtained naturally or comprise a mixture of synthetic and natural DNA constituents, and may consist of different heterologous DOXS gene sections from different organisms. In general, synthetic nucleotide sequences are produced with codons preferred by plants. These codons preferred by plants can be identified from codons with the highest protein frequency which are expressed in most plant



0817/00006

## 22

species of interest. For preparing an expression cassette it is possible to manipulate various DNA fragments in order to obtain a nucleotide sequence which expediently reads in the correct direction and is equipped with a correct reading frame. Adapters  
5 or linkers can be attached to the fragments for connecting the DNA fragments to one another.

It is possible and expedient for the promoter and terminator regions to be provided in the direction of transcription with a  
10 linker or polylinker which contains one or more restriction sites for inserting this sequence. As a rule, the linker has 1 to 10, usually 1 to 8, preferably 2 to 6, restriction sites. The linker generally has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp, inside the regulatory regions. The  
15 promoter may be both native or homologous and foreign or heterologous to the host plant. The expression cassette comprises in the 5'-3' direction of transcription the promoter, a DNA sequence which codes for a DOXS gene, and a region for termination of transcription. Various termination regions are  
20 interchangeable as desired.

It is furthermore possible to employ manipulations which provide appropriate restriction cleavage sites or delete the redundant DNA or restriction cleavage sites. It is possible in relation to  
25 insertions, deletions or substitutions, e.g. transitions and transversions, to use in vitro mutagenesis, primer repair, restriction or ligation. It is possible with suitable manipulations, e.g. restriction, chewing back or filling in overhangs for blunt ends, to provide complementary ends of the fragments  
30 for ligation.

It may be important for success according to the invention inter alia to attach the specific ER retention signal SEKDEL (Schouten, A. et al. Plant Mol. Biol. 30 (1996), 781 - 792), whereby the  
35 average level of expression is tripled or quadrupled. It is also possible to employ other retention signals which naturally occur with plant and animal proteins which are localized within the ER for constructing the cassette.

40 Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, especially of gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 ff) or  
45 functional equivalents.

0817/00006

## 23

An expression cassette may comprise, for example, a constitutive promoter (preferably the CaMV 35 S promoter), the LeB4 signal peptide, the gene to be expressed, and the ER retention signal. The ER retention signal preferably used is the amino acid sequence KDEL (lysine, aspartic acid, glutamic acid, leucine).

The fused expression cassette which codes for a DOXS gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming *Agrobacterium tumefaciens*. *Agrobacteria* transformed with such a vector can then be used in a known manner for transforming plants, in particular crop plants, e.g. tobacco plants, by, for example, bathing wounded leaves or pieces of leaf in a solution of *agrobacteria* and then cultivating in suitable media. The transformation of plants by *agrobacteria* is disclosed inter alia by F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38. Transgenic plants containing a gene, integrated in the expression cassette, for expression of a DOXS gene can be regenerated in a known manner from the transformed cells of the wounded leaves or pieces of leaf.

For transformation of a host plant with a DNA coding for a DOXS, an expression cassette is incorporated as insertion into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described inter alia in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chap. 6/7, pp. 71-119 (1993).

It is possible by using the recombination and cloning techniques cited above to clone the expression cassettes into suitable vectors which make their replication possible, for example in *E. coli*. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Binary vectors able to replicate both in *E. coli* and in *agrobacteria* are particularly suitable.

The invention further relates to the use of an expression cassette comprising a DNA sequence SEQ No. 1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5; SEQ ID No. 1 or SEQ-ID No. 3 and SEQ-ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, or DNA sequences hybridizing with the latter for transforming plants, or cells, tissues or parts of plants. The aim of the use is preferably to

0817/00006

## 24

increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, 5 for expression to take place specifically in the leaves, in the seeds, or other parts of the plant. The present invention further relates to such transgenic plants, to propagation material thereof and to cells, tissues or parts of the plants.

- 10 The expression cassette can in addition be employed for transforming bacteria, cyanobacteria, yeasts, filamentous fungi and algae with the aim of increasing tocopherol, vitamin K, chlorophyll and/or carotenoid production.
- 15 The transfer of foreign genes into the genome of a plant is referred to as transformation. In this connection, the described methods for transforming and regenerating plants from plant tissues or plant cells are utilized for transient or stable transformation. Suitable methods are protoplast transformation by 20 polyethylene glycol-induced DNA uptake, the biolistic method using the gene gun - called the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and gene transfer mediated by *Agrobacterium*. Said processes are described, for example, in 25 B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector 30 which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

*Agrobacteria* transformed with an expression cassette can likewise be used in a known manner for transforming plants, in particular 35 crop plants such as cereals, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species, e.g. by bathing wounded leaves or pieces of leaf in a solution of *agrobacteria* and subsequently cultivating 40 in suitable media.

Functionally equivalent sequences which code for a DOXS gene are sequences which, despite differing in nucleotide sequence, still have the required functions. Functional equivalents thus comprise 45 naturally occurring variants of the sequences described herein, and artificial artificial nucleotide sequences obtained, for

0817/00006

## 25

example, by chemical synthesis and adapted to the codon usage of a plant.

A functional equivalent also means in particular natural or  
5 artificial mutations of an originally isolated sequence coding  
for a DOXS, which still show the required function. Mutations  
comprise substitutions, additions, deletions, transpositions or  
insertions of one or more nucleotide residues. Thus, the present  
10 invention also includes, for example, nucleotide sequences which  
are obtained by modifying the DOXS nucleotide sequence. The aim  
of such a modification may be, for example, to localize further  
the coding sequence present therein or else, for example, to  
insert further restriction enzyme cleavage sites.

15 Functional equivalents are also variants whose function is  
attenuated or enhanced by comparison with the initial gene or  
gene fragment.

Artificial DNA sequences are also suitable as long as they  
20 confer, as described above, the required property, for example of  
increasing the tocopherol content in the plant by overexpression  
of the DOXS gene in crop plants. Such artificial DNA sequences  
can be identified, for example, by back-translation of proteins  
which have been constructed by molecular modelling and have DOXS  
25 activity, or by *in vitro* selection. Particularly suitable coding  
DNA sequences are those which have been obtained by back-  
translation of a polypeptide sequence in accordance with the  
codon usage specific for the host plant. The specific codon usage  
can easily be established by a skilled worker familiar with plant  
30 genetic methods through computer analyses of other, known genes  
in the plant to be transformed.

Further suitable equivalent nucleic acid sequences which should  
be mentioned are sequences which code for fusion proteins, in  
35 which case a plant DOXS polypeptide or a functionally equivalent  
part thereof is a constituent of the fusion protein. The second  
part of the fusion protein can be, for example, another poly-  
peptide with enzymatic activity, or an antigenic polypeptide  
sequence with whose aid it is possible to detect DOXS expression  
40 (e.g. myc tag or his tag). However, this is preferably a  
regulatory protein sequence, e.g. a signal or transit peptide  
which guides the DOXS protein to the required site of action.

However, the invention also relates to the expression products  
45 generated according to the invention, and to fusion proteins  
composed of a transit peptide and a polypeptide with DOXS

0817/00006

26

activity.

Increasing the tocopherol, vitamin K, chlorophyll and/or carotenoid content means for the purpose of the present invention the artificially acquired capability of increased activity in the biosynthesis of these compounds through functional overexpression of the DOXS gene in the plant compared with the plant which has not been genetically modified, for the duration of at least one plant generation.

10

The site of tocopherol biosynthesis is generally the leaf tissue so that leaf-specific expression of the DOXS gene is sensible. However, it is obvious that tocopherol biosynthesis need not be confined to the leaf tissue, but may also take place tissue-specifically in all other parts of the plant - for example in oilbearing seeds.

Constitutive expression of the exogenous DOXS gene is an additional advantage. However, on the other hand, inducible expression may also appear to be desirable.

The effectiveness of expression of the transgenically expressed DOXS gene can be determined, for example, in vitro by shoot meristem propagation. In addition, an alteration in the nature and level of the expression of the DOXS gene and its effect on tocopherol biosynthesis activity can be tested in glasshouse experiments on test plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No.1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ No. 5; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particularly preferred in this connection are transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

Plants for the purpose of the invention are mono- and dicotyledonous plants or algae.

In order to be able to find efficient DOXS inhibitors, it is necessary to provide suitable test systems with which inhibitor/enzyme binding studies can be carried out. For this purpose, for

0817/00006

27

example, the complete cDNA sequence of DOXS from Arabidopsis is cloned into an expression vector (pQE, Qiagen) and overexpressed in *E. coli*.

- 5 The DOXS protein expressed using the expression cassette is particularly suitable for finding inhibitors specific for DOXS.

For this purpose, DOXS can be employed, for example, in an enzyme assay in which the activity of DOXS is determined in the presence  
10 and absence of the active substance to be tested. Comparison of the two activity determinations allows qualitative and quantitative information to be obtained about the inhibitory behavior of the active substance to be tested. Methods for DOXS activity determination are described (Putra et. al., Tetrahedron  
15 Letters 39 (1998), 23-26; Sprenger et al., PNAS 94 (1997), 12857-12862).

The test system according to the invention can be used to examine rapidly and simply a large number of chemical compounds for  
20 inhibitory properties. The method allows reproducible selection, from a large number of substances, specifically of those with high activity in order subsequently to carry out with these substances further, more intensive tests familiar to the skilled worker.

25

It is possible in principle by overexpression of the gene sequence SEQ ID NO: 1 or SEQ ID NO: 3 coding for a DOXS in a plant to achieve increased resistance to DOXS inhibitors. The invention likewise relates to transgenic plants produced in this  
30 way.

The invention further relates to:

- A process for transforming a plant, which comprises  
35 introducing an expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or a DNA sequence hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants.
- 40 - The use of a plant for producing plant DOXS.
- The use of the expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID NO. 3 or a DNA sequence hybridizing with the latter for producing plants with increased  
45 resistance to DOXS inhibitors by enhanced expression of a DNA

0817/00006

28

sequence SEQ ID No. 1 or SEQ ID NO. 3 or a DNA sequence hybridizing with the latter.

- 5 - The use of the DNA sequence SEQ ID No. 1 or SEQ ID NO. 3 or of a DNA sequence hybridizing with the latter for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid content by expression of a DOXS DNA sequence in plants.
- 10 - The use of the expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID NO. 3 or a DNA sequence hybridizing with the latter for producing a test system for identifying DOXS inhibitors.

15 The invention is illustrated by the examples which now follow, but is not confined to these:

#### General cloning methods

- 20 The cloning steps carried out for the purpose of the present invention, such as restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of *E. coli* cells, cultivation of
- 25 bacteria, replication of phages and recombinant DNA sequence analysis were carried out as described in Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6).

- The bacterial strains (*E. coli*, XL-I Blue) used below were
- 30 purchased from Stratagene. The agrobacterium strain used for plant transformation (*Agrobacterium tumefaciens*, C58C1 with the plasmid pGV2260 or pGV3850kann) has been described by Deblaere et al. in (Nucl. Acids Res. 13 (1985) 4777). Alternative possibilities are also to employ the agrobacterium strain LBA4404
  - 35 (Clontech) or other suitable strains. Vectors which can be used for cloning are pUC19 (Yanish-Perron, Gene 33 (1985), 103-119) pBluescript SK- (Stratagene), pGEM-T (Promega), pZero (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66
  - 40 (1990), 221-230).

#### Recombinant DNA sequence analysis

- Recombinant DNA molecules were sequenced using a Licor laser
- 45 fluorescence DNA sequencer (marketed by MWG Biotech, Ebersbach)

0817/00006

## 29

using the Sanger method (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

## Example 1

5

Production of the *Arabidopsis thaliana* DOXS transformation constructs

The *Arabidopsis thaliana* DOXS gene was cloned as described in

10 Mandel et al. (1996) as complete cDNA into the vector pBluescript KS- (Stratagene).

To produce overexpression constructs, a 2.3 kb fragment (designated F-23-C) was isolated via the pBluescript KS- HincII (blunt-end) and SacI cleavage sites. This sequence contains the complete DOXS cDNA from the ATG start codon to the EcoRII cleavage site located 80 bp downstream of the stop codon. This fragment was cloned via the SmaI (blunt-end) and SacI cleavage sites into the pBIN19 vector (Figure 3) (Bevan et al., (1980) which contains the 35S promoter of cauliflower mosaic virus (Franck et al., Cell 21(1), 285-294 (1980)) arranged three times in sequence.

To produce antisense constructs, a region of the 3' end of the 25 cDNA (called F-23-C antisense) was cloned into the abovementioned pBIN19-3X35S vector. Part of the 5' region of the DOXS cDNA in pBluescript KS- was digested via HincII and the DOXS-internal BglII cleavage site, and the resulting fragment was removed. (Figure 4). The BglII cleavage site was filled in by the Klenow 30 fill-in reaction (Klenow polymerase; Roche; after reaction according to manufacturer's protocol) so that a blunt end was produced. The ends which were now compatible (BglII blunt end and HincIII were ligated. The 3' region of the DOXS cDNA was then cloned via KpnI and XbaI (both cleavage sites are located in the 35 polylinker of pBluescript KS-5' and 3' of the DOXS cDNA) in antisense orientation into the pBIN19 vector described above in antisense orientation.

Transformations of *Arabidopsis thaliana* plants with the 40 constructs described above using *Agrobacterium tumefaciens* took place by the vacuum infiltration method (Bent et al., Science 265 (1994), 1856-1860). Several independent transformants were isolated for each construct. Each letter (see Table 1) denotes an independent transformed line. Plants from the T1 generation 45 obtained therefrom were examined for homo- or heterozygosity. Several plants from each line were crossed in order to carry out a segregation analysis. The number in Table 1 corresponds to the



0817/00006

30

individual plant selected for further analyses. Both homo- and heterozygous lines were obtained. The segregation analysis of the resulting lines is shown in Table 1 below:

5 Table 1. Segregation analysis of the transgenic DOXS T2 plants

LINES	SEGREGATION
A9	75%
10 A19	100%
B11	75%
B4	100%
C2	100%
15 D3	75%
D17	100%
E9	75%
E14	100%
F9	75%
20 F14	100%

#### Example 2

#### 25 Isolation of genomic DNA of the bacterium Escherichia coli XL1 Blue

A culture of Escherichia coli XL1 Blue was grown in 300 ml of Luria broth medium at 37°C for 12 hours. The genomic DNA of the bacterium was isolated from this culture by first pelleting it at 30 5 000 revolutions in a Sorvall RC50 fuge. The pellet was then resuspended in 1/30 of the volume of the original culture of lysis buffer (25 mM EDTA, 0.5% SDS; 50 mM Tris HCl, pH 8.0). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and incubated at 70 degrees for 10 minutes. The aqueous 35 phase was then separated from the phenolic in a Heraeus floor centrifuge at 3 500 rev for 15 minutes. The aqueous supernatant was mixed with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride, and the nucleic acids were precipitated at room temperature for 10 minutes. The pellet was then taken up in 40 400 µl of TE/RNase and incubated at 37 degrees for 10 minutes. The solution was again shaken with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the supernatant was precipitated with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride. The pellet was then washed with 80% ethanol and 45 taken up in 400 µl of TE/RNase.

0817/00006

31

## Example 3

Isolation of the DOXS from E. coli

- 5 Oligonucleotides for a PCR were derived from the DOXS DNA sequence (Acc. Number AF035440), and a BamHI restriction cleavage site was attached to them at the 5' end, and an XbaI or another BamHI restriction cleavage site was attached to them at the 3' end. The oligonucleotide at the 5' end comprises the sequence
- 10 5'-ATGGATCCATGAGTTTT-GATATTGCCAAATAC-3' (nucleotides 1-24 of the DNA sequence; in italics) starting with the ATG start codon of the gene, and the oligonucleotide at the 3' end comprises the sequence 5'-ATTCTAGATTATGCCAGCCAGGCCTTG-3' or
- 15 5'-ATGGATCCTTATGCCAGCCAGGCCTTG-3' (nucleotides 1845-1863 of the reverse complementary DNA sequence; in italics) starting with the stop codon of the gene. The PCR reaction with the two BamHI-containing oligonucleotides was carried out with Pfu polymerase (Stratagene GmbH, Heidelberg) in accordance with the manufacturer's information. 500 ng of the genomic DNA from E.
- 20 coli were employed as template. The PCR program was as follows:

5 cycles: 4 sec 94°C, 30 sec 52°C, 2 min 72°C;

5 cycles: 4 sec 94°C, 30 sec 48°C, 2 min 72°C;

25 cycles: 4 sec 94°C, 30 sec 44°C, 2 min 72°C

25

- The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was established by
- 30 sequencing. The fragment was BamHI isolated from the PCR-Script vector and ligated into a correspondingly cut Bin19 vector which additionally contains the transit peptide of potato transketolase downstream of the CaMV 35S as promoter. The transit peptide ensures plastidic localization. The constructs are depicted in
- 35 Figure 5 and 6, and the fragments have the following significance:

- Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic
- 40 virus). Fragment B (259 bp) comprises the transit peptide of transketolase. Fragment E comprises the DOXS gene. Fragment D (192 bp) comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) to terminate transcription.

45

0817/00006

## 32

The PCR reaction with the 5'-BamHI and 3'-XbaI-containing oligonucleotides was carried out with Taq polymerase (Takara, Sosei Co., Ltd.) in accordance with the manufacturer's information. 500 ng of the genomic DNA from *E. coli* were employed as template. The PCR program was as follows:

5 cycles: 4 sec 94°C, 4 sec 50°C, 2 min 30°C  
 5 cycles: 4 sec 94°C, 30 sec 46°C, 2 min 68°C  
 25 cycles: 4 sec 94°C, 30 sec 42°C, 2 min 68°C

10

The fragment was purified using a Gene-Clean kit and ligated into the vector pGemT (Promega GmbH, Mannheim). It was cloned as BamHI/XbaI fragment into a correspondingly cut pBin19AR vector downstream of the CaMV 35S promoter. The sequence was checked by sequencing (SEQ-ID No. 3). This revealed two non-conservative base exchanges which, compared with the published sequence, lead to a change in amino acid 152 (asparagine) to valine and amino acid 330 (cysteine) to tryptophan.

## 20 Example 4

Detection of increased amounts of DOXS RNA in transgenic plants

Total RNA from 15-day old seedlings of various transgenic lines possessing the DOXS overexpression construct was extracted by the method of Logeman et al., Anal. Biochem. 163, 16-20 (1987), fractionated in a 1.2% agarose gel, transferred to filters and hybridized with a 2.1 kb long DOXS fragment as probe (Figure 7).

## 30 Example 5

Detection of increased amounts of DOXS protein in transgenic plants

Total protein (Figure 8) from 15-day old seedlings of various independent transgenic plants possessing the DOXS overexpression construct was isolated and detected in a Western analysis using a polyclonal anti-DOXS antibody (IgG) (Figure 9).

## 40 Example 6

Measurement of the carotenoid and chlorophyll contents

The total amounts of carotenoids and chlorophylls were determined as described by Lichtenthaler and Wellburn (1983) using 100% acetone extracts. The results of the multiple measurements of the

0817/00006

## 33

transgenic lines possessing the DOXS overexpression construct are shown in Table 2 below.

Table 2: Total carotenoid and chlorophyll contents of transgenic  
5 DOXS lines

	LINE	% TOTAL CHLOROPHYLLS	% TOTAL CAROTENOIDS
	clal mutant	5	5
10	Wild type	100	100
	B-4	86	89
	B-11	84	90
	C-2	98	107
15	D-3	128	135
	D-17	136	149
	E-14	121	139
	F-7	80	90
20	F-14	85	107

## Example 7

## Transformation of oilseed rape

- 25 The production of transgenic oilseed rape plants is based on a protocol of Bade, JB and Damm, B (in Gene, Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38), in which the composition of the media used are also stated. The transformations took place with
- 30 the agrobacterium strain LBA4404 (Clontech). The binary vectors used were the pBIN19 constructs with the complete DOXS cDNA already described above. The NOS terminator sequence in these pBIN vectors was replaced by the OCR terminator sequence. Brassica napus seeds were surface-sterilized with 70% (v/v)
- 35 ethanol, washed in H<sub>2</sub>O at 55°C for 10 min, incubated in 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Twenn 20) for 20 min and washed six times with sterile H<sub>2</sub>O for 20 min each time. The seeds were dried on filter paper for three days and 10-15 seeds were induced to germinate in a glass flask with
- 40 15 ml of germination medium. The roots and apices were removed from several seedlings (about 10 cm in size), and the remaining hypocotyls were cut into pieces about 6 mm long. The approx. 600 explants obtained in this way are washed with 50 ml of basal medium for 30 min and transferred into a 300 ml flask. After
- 45 addition of 100 ml of callus induction medium, the cultures were incubated at 100 rpm for 24 h.

0817/00006

## 34

An overnight culture of the agrobacterium strain was set up in LB with kanamycin (20 mg/l) at 29°C, and 2 ml of this were incubated in 50 ml of LB without kanamycin at 29°C for 4 h until the OD<sub>600</sub> was 0.4-0.5. After pelleting of the culture at 2 000 rpm for 5 25 min, the cell pellet was resuspended in 25 ml of basal medium. The concentration of the bacteria in the solution was adjusted to an OD<sub>600</sub> of 0.3 by adding further basal medium.

The callus induction medium was removed from the oilseed rape explants using sterile pipettes, 50 ml of agrobacterium solution were added and, after cautious mixing, incubated for 20 min. The agrobacteria suspension was removed, the oilseed rape explants were washed with 50 ml of callus induction medium for 1 min and then 100 ml of callus induction medium were added. The cocultivation was carried out on a rotary shaker at 100 rpm for 24 h. The cocultivation was stopped by removing the callus induction medium, and the explants were washed twice for 1 min each time with 25 ml and twice for 60 min with 100 ml each time of washing medium at 100 rpm. The washing medium with the explants was transferred into 15 cm Petri dishes, and the medium was removed using sterile pipettes. For regeneration, in each case 20-30 explants were transferred into 90 mm Petri dishes which contained 25 ml of shoot-induction medium with kanamycin. The Petri dishes were sealed with 2 layers of Leukopor and incubated at 25°C and with 2000 lux in 16/8 h photoperiods. The calli which developed was transferred every 12 days to fresh Petri dishes with shoot-induction medium. All further steps for regenerating whole plants were carried out as described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38).

## Example 8

## 35 Increasing tocopherol biosynthesis in oilseed rape

The DOXS cDNA (SEQ-ID No. 1) was provided with a CaMV 35S promoter and over-expressed in oilseed rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The  $\alpha$ -tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the  $\alpha$ -tocopherol concentration was increased by comparison with the untransformed plant.

0817/00006

## 35

## Example 9

Detection of the expression of DOXS from *E. coli* in transgenic tobacco plants

5

Leaf disks with a diameter of 0.9 cm were taken from completely unfolded leaves of plants containing the construct pBinAR HPPD-DOXS, and were frozen in liquid nitrogen. The leaf material was homogenized in an HEPES-KOH buffer containing proteinase inhibitors, and the protein concentration was determined from the extract using the Bio-Rad protein assay in accordance with the manufacturer's information. 45 µg of protein from each extract were mixed with one volume of loading buffer (Laemmli, 1970) and incubated at 95°C for 5 min. The proteins were then fractionated on a 12.5 percent SDS-PAGE gel. The proteins were then transferred by means of semi-dry electroblots to Porablot membrane (Machery und Nagel). Detection of the DOXS protein took place using a rabbit antibody against *E. coli* DOXS. The color reaction is based on the binding of a secondary antibody and of an alkaline phosphatase which converts NBT/BCIP into a dye. Secondary antibody and alkaline phosphatase were obtained from Pierce, and the procedure was in accordance with the manufacturer's information.

Figure 10 shows the detection of the DOXS protein in leaves of transgenic plants. 1: marker; 2: plant 10; 3:62; 4: 63; 5: 69; 7:71; 8:112; 9:113; 10:116; 11:WT1; 12:WT2; 13:100 ng of recombinant protein; 14:50 ng of recombinant protein; 15: 10 ng of recombinant protein.

30

## Example 10

Cloning of an HPPD gene from *Streptomyces avermitilis* U11864

Isolation of genomic DNA of the bacterium *Streptomyces avermitilis* U11864:

A culture of *Streptomyces avermitilis* U11864 was grown in 300 ml of YEME medium (5 g of malt extract, 2 g of yeast extract, 2 g of glucose) at 28°C for 96 h. The genomic DNA of the bacterium was isolated from this culture by pelleting it initially at 5000 rev in a Sorvall RC5C fuge. The pellet was then resuspended in 1/30 of the volume of lysis buffer (25 mM EDTA, 0.5% SDS, 50 mM Tris-HCl, pH 8.0). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and incubated at 70°C for 10 minutes. The aqueous phase was then separated from the phenolic in a Heraeus floor centrifuge at 3 500 rev for 15 minutes. The aqueous

0817/00006

## 36

supernatant was mixed with 2.5 volumes of ethanol in 1/10 volume of 8 M lithium chloride, and the nucleic acids were precipitated at room temperature for 10 minutes. The pellet was then taken up in 400 µl of TE/RNase and incubated at 37 degrees for 10 minutes.

5 The solution was again shaken with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the supernatant was precipitated with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride. The pellet was then washed with 80% ethanol and taken up in 400 µl of TE/RNase.

10

Oligonucleotides were derived for a PCR from the DNA sequence of the HPPD from *Streptomyces avermitilis* (Denoya et al., 1944; Acc. Number U11864), and a BamHI restriction cleavage site was attached to the 5' end of them and an XbaI restriction cleavage  
15 site was attached at the 3' end of them. The oligonucleotide at the 5' end comprises the sequence 5'-GGATCCAGCGGACAAGCCAAC-3' (37 to 55 bases distant from the ATG in the 5' direction; in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-TCTAGATTATGCCAGCCAGGCCTTG-3' (nucleotides 1845-1863  
20 of the reverse complementary DNA sequence; in italics).

The PCR reaction was carried out with Pfu polymerase (Stratagene GmbH, Heidelberg) in accordance with the manufacturer's information. 400 ng of the genomic DNA was employed as pattern.

25 The PCR program was as follows:

5 cycles: 4 sec 94°C, 30 sec 54°C, 2 min 72°C  
5 cycles: 4 sec 94°C, 30 sec 52°C, 2 min 72°C  
25 cycles: 4 sec 94°C, 30 sec 50°C, 2 min 72°C

30

The fragment was purified by means of a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by

35 sequencing. This revealed that the isolated gene codes for an additional amino acid. It contains the three bases TAC (coding for tyrosine) in front of nucleotide N429 in the quoted sequence (Denoya et al., 1994).

40 The fragment was isolated by a BamHI and XbaI digestion from the vector and ligated into a correspondingly cut Bin19AR vector downstream of the CaMV 35S promoter for expression of the gene in the cytosol. The gene was isolated as BamHI fragment from the same PCR-Script vector and was ligated into a correspondingly cut  
45 pBin19 vector which additionally comprises the transit peptide of the potato plastidic transketolase downstream of the CaMV 35S promoter. The transit peptide ensures the plastidic localization.

0817/00006

## 38

The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by sequencing. It was cut as BamHI fragment out of the vector PCR-Script and ligated into a correspondingly cut pBinAR vector which additionally contains the transit peptide of transketolase for introducing the gene product into the plastids. The result was the plasmid pBinAR-TP-HPPD (Figure 12).

10

For the cloning, the 35S promoter, the transketolase transit peptide, the HPPD gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al. 1984) for termination of transcription was isolated from the plasmid

15 pBinAR-TP-HPPD by PCR. A HindIII cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the 5' region of the promoter (in italics) is 5'-ATAAGCTTCATGGAGTCAAA-GATTCAAATAGA-3', and that of the 20 oligonucleotide which anneals onto the termination sequence (in italics) is 5'-ATAAGCTTGGACAATCAGTAAATTGAACGGAG-3'. The resulting fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, 25 Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred as HindIII fragment from this PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984, Nucleic Acids Res. 12, 8711-8721).

30 The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated by PCR from the plasmid pBinAR-TP-DOXS. An EcoRI cleavage site was attached to each of

35 the oligonucleotides for the promoter and terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTCCATGGAGTCAAAGATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTCGGACAATCAGTAAATTGAA-CGGAG-3'.

40 The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by sequencing (SEQ ID No. 3). It was transferred as EcoRI fragment 45 from the PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984).



0817/00006

## 39

It was transferred as XbaI fragment from the PCR-Script vector into the correspondingly cut vector which, as described above, already contains the HPPD sequence. The result was the construct pBinAR-HPPD-DOXS (Figure 13), whose fragments have the following  
5 significance:

Fragment A (529 bp) comprises the 35S promoter of the cauliflower mosaic virus (nucleotides 6909 to 7437). Fragment B comprises the transit peptide of plastidic transketolase. Fragment C comprises  
10 the HPPD gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment E comprises the DOXS gene.

## 15 Example 12

Production of transgenic tobacco plants  
(*Nicotiana tabacum* L. cv. Samsun NN)

20 Transgenic tobacco plants having an altered prenyl lipid content were produced by transforming tobacco leaf disks with DOXS and HPPD sequences. To transform tobacco plants, 10 ml of an *Agrobacterium tumefaciens* overnight culture which had grown under selection were centrifuged, the supernatant was discarded and the  
25 bacteria were resuspended in the same volume of antibiotic-free medium. Leaf disks from sterile plants (diameter about 1 cm) were bathed in this bacterial suspension in a sterile Petri dish. The leaf disks were then placed on MS medium (Murashige and Skoog, *Physiol. Plant* (1962) 15, 473) with 2% sucrose and 0.8% Bacto  
30 agar in Petri dishes. After incubation in the dark at 25°C for 2 days, they were transferred to MS medium with 100 mg/l kanamycin, 500 mg/l Claforan, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid (NAA), 1.6% glucose and 0.8% Bacto  
35 agar, and the cultivation was continued (16 hours of light/ 8 hours of dark). Growing shoots were transferred to hormone-free MS medium with 2% sucrose, 250 mg/l Claforan and 0.8% Bacto agar.

## Example 13

40 Production of transgenic oilseed rape plants (*Brassica napus*)

The production of transgenic oilseed rape plants having an altered prenyl lipid content was based on a protocol by  
Bade, J.B. and Damm, B. (in *Gene Transfer to Plants*, Potrykus, I.  
45 and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag,

0817/00006

## 40

1995, 30-38), in which the compositions of the media and buffers used are also indicated.

The transformations took place with the *Agrobacterium tumefaciens* strain LBA4404 (Clontech GmbH, Heidelberg). The binary vectors used were the binary constructs already described above with the total cDNAs of DOXS and HPPD. In all the binary vectors used here, the NOS terminator sequence was replaced by the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Brassica napus seeds were surface-sterilized with 70% (v/v) ethanol, washed in H<sub>2</sub>O at 55°C for 10 min, incubated in 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Tween 20) for 20 min and washed six times with sterile H<sub>2</sub>O for 20 min each time. The seeds were dried on filter paper for three days and 10-15 seeds were induced to germinate in a glass flask with 15 ml of germination medium. The roots and apices were removed from several seedlings (about 10 cm in size), and the remaining hypocotyls were cut into pieces about 6 mm long. The approx. 600 explants obtained in this way are washed with 50 ml of basal medium for 30 min and transferred into a 300 ml flask. After addition of 100 ml of callus induction medium, the cultures were incubated at 100 rpm for 24 h.

An overnight culture of the agrobacterium strain was set up in Luria Broth medium with kanamycin (20 mg/l) at 29°C, and 2 ml of this were incubated in 50 ml of Luria Broth medium without kanamycin at 29°C for 4 h until the OD<sub>600</sub> was 0.4-0.5. After pelleting of the culture at 2 000 rpm for 25 min, the cell pellet was resuspended in 25 ml of basal medium. The concentration of the bacteria in the solution was adjusted to an OD<sub>600</sub> of 0.3 by adding further basal medium.

The callus induction medium was removed from the oilseed rape explants using sterile pipettes, 50 ml of agrobacterium solution were added and, after cautious mixing, incubated for 20 min. The agrobacteria suspension was removed, the oilseed rape explants were washed with 50 ml of callus induction medium for 1 min and then 100 ml of callus induction medium were added. The cocultivation was carried out on a rotary shaker at 100 rpm for 24 h. The cocultivation was stopped by removing the callus induction medium, and the explants were washed twice for 1 min each time with 25 ml and twice for 60 min with 100 ml each time of washing medium at 100 rpm. The washing medium with the explants was transferred into 15 cm Petri dishes, and the medium was removed using sterile pipettes.

0817/00006

## 41

For regeneration, in each case 20-30 explants were transferred into 90 mm Petri dishes which contained 25 ml of shoot-induction medium with kanamycin. The Petri dishes were sealed with 2 layers of Leukopor and incubated at 25°C and with 2 000 lux in 5 photoperiods of 16 hours of light/8 hours of dark. The calli which developed were transferred every 12 days to fresh Petri dishes with shoot-induction medium. All further steps for regenerating whole plants ~~were~~ carried out as described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and 10 Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38).

## Example 14

## 15 Increasing tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ-ID No. 3) and of HPPD (SEQ-ID No. 5) was provided with a CaMV35S promoter and overexpressed in oilseed rape using the 35S promoter. In parallel with this, the 20 seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rape seed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The  $\alpha$ -tocopherol content of the whole plant and of the seeds of the plant was then determined. In all 25 cases, the  $\alpha$ -tocopherol concentration was increased by comparison with the untransformed plant.

## Example 15

## 30 Cloning of a GGPPOR gene from Arabidopsis thaliana

Isolation of total RNA from completely unfolded leaves of Arabidopsis thaliana:

35 Completely unfolded leaves of Arabidopsis thaliana were harvested and frozen in liquid nitrogen. The material was then powdered in a mortar and taken up in Z6 buffer (8 M guanidium hydrochloride, 20 mM MES, 20 mM EDTA pH 7.0). The suspension was transferred into reaction vessels and shaken with one volume of phenol/ 40 chloroform/isoamyl alcohol 25:24:1). After centrifugation at 15 000 rpm for 10 minutes, the supernatant was transferred into a new reaction vessel, and the RNA was precipitated with 1/20 volumes of 1N acetic acid and 0.7 volume of ethanol (absolute). After renewed centrifugation, the pellet was first 45 washed with 3M sodium acetate solution and, after a further centrifugation, in 70% ethanol. The pellet was then dissolved in

0817/00006

## 43

confers on plants resistance to the antibiotic hygromycin and is thus suitable for superinfection of plants with kanamycin resistance. Since the plastid transit peptide of GGPPOR was also cloned, the protein ought to be transported into the plastids in transgenic plants. The construct is depicted in Figure 14. The fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment F comprises the gene of GGPPOR including the intrinsic plastid transit sequence.

## 15 Example 16

Production of constructs for transformation of plants with DOXS and GGPPOR sequences

To produce plants which are transgenic for DOXS and GGPPOR, a binary vector comprising both gene sequences was manufactured (Figure 15). The GGPPOR gene with the intrinsic plastidic localization sequence was cloned (as described in Example 15) as BamHI/SalI fragment into the correspondingly cut vector pBinAR-Hyg. The DOXS gene was cloned as BamHI fragment as described in Example 3. The vector pBinAR-Hyg contains the 35S promoter of cauliflower mosaic virus and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. This plasmid confers on plants resistance to the antibiotic hygromycin and is thus suitable for superinfection of plants with kanamycin resistance.

The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-DOXS by PCR. An EcoRI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTCCATGGAGTCAAAGATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTCGGACAATCAGTAAATTGAACGGAG-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by

0817/00006

44

sequencing. It was transferred from the PCR-Script vector as EcoRI fragment into the correspondingly cut vector pBin19 (Bevan, Nucleic Acids Res. 12 (1984), 8711-8721).

5 The 35S promoter, the GGPPOR gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinARHyg-GGPPOR by PCR. An XbaI cleavage site was attached in each case to the oligonucleotides for the promoter  
10 and the terminator. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATTCTAGACATGGAGTCAAA-GATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATTCTAGAGGACAA-TCAGTAAATTGAACGGAG-3'. The fragment  
15 was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information onto the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as XbaI fragment into the  
20 correspondingly cut vector which already contained, as described above, the DOXS sequence. The result was the construct pBinAR-DOXS-GGPPOR (Figure 15), whose fragments have the following significance:

25 Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B comprises the transit peptide of the plastidic transketolase. Fragment E comprises the DOXS gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of  
30 the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment F comprises the GGPPOR gene including the intrinsic plastid transit sequence.

#### Example 17

35

Production of constructs for transformation of plants with DOXS, GGPPOR and HPPD DNA sequences

To produce plants which are transgenic for DOXS, GGPPOR and HPPD,  
40 a binary vector containing all three gene sequences was manufactured (Figure 16). The GGPPOR gene was provided with the intrinsic plastidic localization sequence (as described in Example 15). The pBinAR-Hyg vector used confers on plants resistance to the antibiotic hygromycin and is thus suitable for  
45 superinfection of plants with kanamycin resistance.

0817/00006

## 45

To clone HPPD into vectors which additionally contain another cDNA, oligonucleotides were derived for a PCR, and a BamHI restriction cleavage site was attached to them at the 5' end and 3' end. The oligonucleotide at the 5' end comprises the sequence 5 5'-GGATCCTCCAGCGGACAAGCCAAC-3' (nucleotides 37 to 55 distant from ATG in the 5' direction; in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-ATGGATCCCGCGCCGCTACAGGTG-3' (ending with base pair 1140 of the coding sequence, starting 8 base pairs 3' of the TAG stop 10 codon; in italics). The PCR reaction was carried out with Tli polymerase from Promega GmbH, Mannheim in accordance with the manufacturer's information. 10 ng of the plasmid pBinAR-HPPD were employed as template. The PCR program was as follows:

15 5 cycles: 94°C 4 sec, 68°C 30 sec, 72°C 2 min  
 5 cycles: 94°C 4 sec, 64°C 30 sec, 72°C 2 min  
 25 cycles: 94°C 4 sec, 60°C 30 sec, 72°C 2 min

The fragment was purified using a Gene-Clean kit (Dianova GmbH, 20 Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was cut out of the vector PCR-Script as BamHI fragment and ligated into a correspondingly cut pBinAR vector 25 which additionally contains the transit peptide of transketolase for introducing the gene product into plastids. The result was the plasmid pBinAR-TP-p-HPPD.

For the cloning, the 35S promoter, the transketolase transit 30 peptide, the p-HPPD gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al. 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-p-HPPD by PCR. A HindIII cleavage site was attached in each case to the oligonucleotides for the promoter and the 35 terminator. The sequence of the oligonucleotide which anneals onto the 5' region of the promoter (in italics) is 5'-ATAAGCTTCATGGAGTCAAA-GATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the termination sequence (in italics) is 5'-ATAAGCTTGGAC-AATCAGTAAATTGAACGGAG-3'. The 40 resulting fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred as HindIII fragment from this 45 PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984, Nucleic Acids Res. 12, 8711-8721).

0817/00006

## 46

The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-DOXS by PCR. An EcoRI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTCATGGAGTCAAAGATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTCGGACAATCAGTAAATTGAACGGAG-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as EcoRI fragment into the correspondingly cut vector which already contained the HPPD sequence as described above.

The 35S promoter, the GGPPOR gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinARHyg-GGPPOR by PCR. An XbaI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATTCTAGACATGGAGTCAAAGATTCAAATAGA-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATTCTAGAGGACAA-TCAGTAAATTGAACGGAG-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as XbaI fragment into the correspondingly cut vector which already contained the HPPD and DOXS sequences as described above. The result was the construct pBinAR-DOXS-GGPPOR-HPPD (Figure 16), whose fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B comprises the transit peptide of the plastidic transketolase. Fragment C comprises the HPPD gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment E comprises the DOXS gene. Fragment F

0817/00006

47

comprises the GGPPOR gene including the intrinsic plastid transit sequence.

## Example 18

5

## Increasing tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ ID No. 3) and of GGPPOR (SEQ ID No. 7) was provided with a CaMV35S promoter and overexpressed in rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The  $\alpha$ -tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the  $\alpha$ -tocopherol concentration was increased by comparison with the untransformed plant.

## Example 19

20

## Increasing the tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ ID No. 3), of HPPD (SEQ ID No. 5) and of GGPPOR (SEQ-ID No. 7) was provided with a CaMV35S promoter and overexpressed in rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The  $\alpha$ -tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the  $\alpha$ -tocopherol concentration was increased by comparison with the untransformed plant.

35

40

45



We claim:

1. The use of DNA sequences coding for a  
5 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) for producing  
plants with increased tocopherol, vitamin K, chlorophyll  
and/or carotenoid contents.
2. The use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or of  
10 a DNA sequence which hybridizes with the latter and codes for  
a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) for  
producing plants with increased content of tocopherols,  
vitamin K, chlorophylls and/or carotenoids.
- 15 3. The use of DNA sequences coding for a  
1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and coding for  
a p-hydroxyphenylpyruvate dioxygenase (HPPD) for producing  
plants with increased tocopherol, vitamin K, chlorophyll  
and/or carotenoid contents.  
20
4. The use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of  
a DNA sequence SEQ ID No. 5 or of a DNA sequence which  
hybridizes with the latter and codes for a  
1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a  
25 p-hydroxyphenylpyruvate dioxygenase for producing plants with  
increased content of tocopherols, vitamin K, chlorophylls  
and/or carotenoids.
5. The use of DNA sequences coding for a  
30 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and coding for  
a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for  
producing plants with increased tocopherol, vitamin K,  
chlorophyll and/or carotenoid contents.
- 35 6. The use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of  
a DNA sequence SEQ ID No. 7 or of a DNA sequence which  
hybridizes with the latter and codes for a  
1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a  
geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for  
40 producing plants with increased tocopherol, vitamin K,  
chlorophyll and/or carotenoid contents.
7. The use of DNA sequences coding for a  
1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and coding for  
45 a hydroxyphenylpyruvate dioxygenase (HPPD) and coding for a  
geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for

## 49

producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents.

8. The use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of  
5 a DNA sequence SEQ ID No. 5 and of a DNA sequence SEQ ID No. 7 or of a DNA sequence which hybridizes with the latter and codes for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS), a hydroxyphenylpyruvate dioxygenase (HPPD) and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for  
10 producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids.
9. A process for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, which  
15 comprises expressing a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or a DNA sequence which hybridizes with the latter in plants.
10. A process for producing plants with increased tocopherol,  
20 vitamin K, chlorophyll and/or carotenoid contents, which comprises expressing a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and a DNA sequence SEQ ID No. 5 or DNA sequences which hybridize with the latter in plants.
- 25 11. A process for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, which comprises expressing a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and a DNA sequence SEQ ID No. 7 or DNA sequences which hybridize with the latter in plants.  
30
12. A process for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, which  
35 comprises expressing DNA sequences SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences which hybridize with the latter in plants.
13. A process for transforming a plant, which comprises  
40 introducing an expression cassette comprising a promoter and a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 into a plant cell, into callus tissue, a whole plant or protoplasts of plant cells.
14. A process for transforming a plant, which comprises  
45 introducing an expression cassette comprising a promoter and DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5

0817/00006

50

into a plant cell, into callus tissue, a whole plant or protoplasts of plant cells.

15. A process for transforming a plant, which comprises  
5 introducing an expression cassette comprising a promoter and DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 into a plant cell, into callus tissue, a whole plant or protoplasts of plant cells.
- 10 16. A process for transforming a plant, which comprises introducing an expression cassette comprising a promoter and DNA sequences SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 into a plant cell, into callus tissue, a whole plant or protoplasts of plant cells.
- 15 17. A process for transforming plants as claimed in claim 13-16, wherein the transformation takes place with the aid of the strain *Agrobacterium tumefaciens*, of electroporation or of the particle bombardment method.
- 20 18. A plant transformed with an expression cassette as set forth in claim 13-16.
- 25 19. A plant as claimed in claim 18 selected from the group of soybean, canola, barley, oats, wheat, oilseed rape, corn or sunflower.
- 30 20. The use of SEQ ID No. 1 or SEQ-ID No. 3 for producing a test system for identifying DOXS inhibitors
- 35 21. A test system based on the expression of an expression cassette as set forth in claim 13 for identifying DOXS inhibitors.
- 40 22. The use of a plant comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or a DNA sequence which hybridize with the latter for producing plant and bacterial DOXS.
- 45

WO 00/08169

PCT/EP99/05467

## SEQUENZPROTOKOLL

&lt;110&gt; SunGene GmbH &amp; Co.KG&amp;A

<120> DNA-Sequenz kodierend fuer eine  
1-Deoxy-D-xylulose-5-phosphat Synthase

&lt;130&gt; 0050/49249

&lt;140&gt; 0817 - 00006

&lt;141&gt; 1999-08-04

&lt;160&gt; 8

&lt;170&gt; PatentIn Vers. 2.0

&lt;210&gt; 1

&lt;211&gt; 2458

&lt;212&gt; DNA

&lt;213&gt; Arabidopsis thaliana

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(2154)

&lt;400&gt; 1

atg gct tct tct gca ttt gct ttt cct tct tac ata ata acc aaa gga	48
Met Ala Ser Ser Ala Phe Ala Phe Pro Ser Tyr Ile Ile Thr Lys Gly	

1	5	10	15
---	---	----	----

gga ctt tca act gat tct tgt aaa tca act tct ttg tct tct tct aga	96
Gly Leu Ser Thr Asp Ser Cys Lys Ser Thr Ser Leu Ser Ser Ser Arg	

20	25	30
----	----	----

tct ttg gtt aca gat ctt cca tca cca tgt ctg aaa ccc aac aac aat	144
Ser Leu Val Thr Asp Leu Pro Ser Pro Cys Leu Lys Pro Asn Asn Asn	

35	40	45
----	----	----

tcc cat tca aac aga aga gca aaa gtg tgt gct tca ctt gca gag aag	192
Ser His Ser Asn Arg Arg Ala Lys Val Cys Ala Ser Leu Ala Glu Lys	

50	55	60
----	----	----

ggt gaa tat tat tca aac aga cca cca act cca tta ctt gac act att	240
Gly Glu Tyr Tyr Ser Asn Arg Pro Pro Thr Pro Leu Leu Asp Thr Ile	

65	70	75	80
----	----	----	----

aac tac cca atc cac atg aaa aat ctt tct gtc aag gaa ctg aaa caa	288
Asn Tyr Pro Ile His Met Lys Asn Leu Ser Val Lys Glu Leu Lys Gln	

WO 00/08169

PCT/EP99/05467

85	90	95	
ctt tct gat gag ctg aga tca gac gtg atc ttt aat gtg tcg aaa acc			336
Leu Ser Asp Glu Leu Arg Ser Asp Val Ile Phe Asn Val Ser Lys Thr			
100	105	110	
ggt gga cat ttg ggg tca agt ctt ggt gtt gtg gag ctt act gtg gct			384
Gly Gly His Leu Gly Ser Ser Leu Gly Val Val Glu Leu Thr Val Ala			
115	120	125	
ctt cat tac att ttc aat act cca caa gac aag att ctt tgg gat gtt			432
Leu His Tyr Ile Phe Asn Thr Pro Gln Asp Lys Ile Leu Trp Asp Val			
130	135	140	
ggt cat cag tct tat cct cat aag att ctt act ggg aga aga gga aag			480
Gly His Gln Ser Tyr Pro His Lys Ile Leu Thr Gly Arg Arg Gly Lys			
145	150	155	160
atg cct aca atg agg caa acc aat ggt ctc tct ggt ttc acc aaa cga			528
Met Pro Thr Met Arg Gln Thr Asn Gly Leu Ser Gly Phe Thr Lys Arg			
165	170	175	
gga gag agt gaa cat gat tgc ttt ggt act gga cac agc tca acc aca			576
Gly Glu Ser Glu His Asp Cys Phe Gly Thr Gly His Ser Ser Thr Thr			
180	185	190	
ata tct gct ggt tta gga atg gcg gta gga agg gat ttg aag ggg aag			624
Ile Ser Ala Gly Leu Gly Met Ala Val Gly Arg Asp Leu Lys Gly Lys			
195	200	205	
aac aac aat gtg gtt gct gtg att ggt gat ggt gcg atg acg gca gga			672
Asn Asn Asn Val Val Ala Val Ile Gly Asp Gly Ala Met Thr Ala Gly			
210	215	220	
cag gct tat gaa gcc atg aac aac gcc gga tat cta gac tct gat atg			720
Gln Ala Tyr Glu Ala Met Asn Asn Ala Gly Tyr Leu Asp Ser Asp Met			
225	230	235	240
att gtg att ctt aat gac aac aag caa gtc tca tta cct aca gct act			768
Ile Val Ile Leu Asn Asp Asn Lys Gln Val Ser Leu Pro Thr Ala Thr			
245	250	255	
ttg gat gga cca agt cca cct gtt ggt gca ttg agc agt gct ctt agt			816
Leu Asp Gly Pro Ser Pro Pro Val Gly Ala Leu Ser Ser Ala Leu Ser			
260	265	270	
cgg tta cag tct aac ccg gct ctc aga gag ttg aga gaa gtc gca aag			864
Arg Leu Gln Ser Asn Pro Ala Leu Arg Glu Leu Arg Glu Val Ala Lys			

WO 00/08169

PCT/EP99/05467

275	280	285	
ggt atg aca aag caa ata ggc gga cca atg cat cag ttg gcg gct aag			912
Gly Met Thr Lys Gln Ile Gly Gly Pro Met His Gln Leu Ala Ala Lys			
290	295	300	
gta gat gtg tat gct cga gga atg ata agc ggt act gga tcg tca ctg			960
Val Asp Val Tyr Ala Arg Gly Met Ile Ser Gly Thr Gly Ser Ser Leu			
305	310	315	320
ttt gaa gaa ctc ggt ctt tac tat att ggt cca gtt gat ggg cac aac			1008
Phe Glu Glu Leu Gly Leu Tyr Tyr Ile Gly Pro Val Asp Gly His Asn			
325	330	335	
ata gat gat ttg gta gcc att ctt aaa gaa gtt aag agt acc aga acc			1056
Ile Asp Asp Leu Val Ala Ile Leu Lys Glu Val Lys Ser Thr Arg Thr			
340	345	350	
aca gga cct gta ctt att cat gtg gtg acg gag aaa ggt cgt ggt tat			1104
Thr Gly Pro Val Leu Ile His Val Val Thr Glu Lys Gly Arg Gly Tyr			
355	360	365	
cct tac gcg gag aga gct gat gac aaa tac cat ggt gtt gtg aaa ttt			1152
Pro Tyr Ala Glu Arg Ala Asp Asp Lys Tyr His Gly Val Val Lys Phe			
370	375	380	
gat cca gca acg ggt aga cag ttc aaa act act aat gag act caa tct			1200
Asp Pro Ala Thr Gly Arg Gln Phe Lys Thr Thr Asn Glu Thr Gln Ser			
385	390	395	400
tac aca act tac ttt gcg gag gca tta gtc gca gaa gca gag gta gac			1248
Tyr Thr Thr Tyr Phe Ala Glu Ala Leu Val Ala Glu Ala Glu Val Asp			
405	410	415	
aaa gat gtg gtt gcg att cat gca gcc atg gga ggt gga acc ggg tta			1296
Lys Asp Val Val Ala Ile His Ala Ala Met Gly Gly Gly Thr Gly Leu			
420	425	430	
aat ctc ttt caa cgt cgc ttc cca aca aga tgt ttc gat gta gga ata			1344
Asn Leu Phe Gln Arg Arg Phe Pro Thr Arg Cys Phe Asp Val Gly Ile			
435	440	445	
gcg gaa caa cac gca gtt act ttt gct gcg ggt tta gcc tgt gaa ggc			1392
Ala Glu Gln His Ala Val Thr Phe Ala Ala Gly Leu Ala Cys Glu Gly			
450	455	460	
ctt aaa ccc ttc tgt gca atc tat tcg tct ttc atg cag cgt gct tat			1440
Leu Lys Pro Phe Cys Ala Ile Tyr Ser Ser Phe Met Gln Arg Ala Tyr			

WO 00/08169

PCT/EP99/05467

465	470	475	480	
gac cag gtt gtc cat gat gtt gat ttg caa aaa tta ccg gtg aga ttt				1488
Asp Gln Val Val His Asp Val Asp Leu Gln Lys Leu Pro Val Arg Phe				
	485	490	495	
gca atg gat aga gct gga ctc gtt gga gct gat ggt ccg aca cat tgt				1536
Ala Met Asp Arg Ala Gly Leu Val Gly Ala Asp Gly Pro Thr His Cys				
	500	505	510	
gga gct ttc gat gtg aca ttt atg gct tgt ctt cct aac atg ata gtg				1584
Gly Ala Phe Asp Val Thr Phe Met Ala Cys Leu Pro Asn Met Ile Val				
	515	520	525	
atg gct cca tca gat gaa gca gat ctc ttt aac atg gtt gca act gct				1632
Met Ala Pro Ser Asp Glu Ala Asp Leu Phe Asn Met Val Ala Thr Ala				
	530	535	540	
gtt gcg att gat gat cgt cct tct tgt ttc cgt tac cct aga ggt aac				1680
Val Ala Ile Asp Asp Arg Pro Ser Cys Phe Arg Tyr Pro Arg Gly Asn				
	545	550	555	560
ggg att gga gtt gca tta cct ccc gga aac aaa ggt gtt cca att gag				1728
Gly Ile Gly Val Ala Leu Pro Pro Gly Asn Lys Gly Val Pro Ile Glu				
	565	570	575	
att ggg aaa ggt aga att tta aag gaa gga gag aga gtt gcg ttg ttg				1776
Ile Gly Lys Gly Arg Ile Leu Lys Glu Gly Glu Arg Val Ala Leu Leu				
	580	585	590	
ggg tat ggc tca gca gtt cag agc tgt tta gga gcg gct gta atg ctc				1824
Gly Tyr Gly Ser Ala Val Gln Ser Cys Leu Gly Ala Ala Val Met Leu				
	595	600	605	
gaa gaa cgc gga tta aac gta act gta gcg gat gca cgg ttt tgc aag				1872
Glu Glu Arg Gly Leu Asn Val Thr Val Ala Asp Ala Arg Phe Cys Lys				
	610	615	620	
cca ttg gac cgt gct ctc att cgc agc tta gct aag tcg cac gag gtt				1920
Pro Leu Asp Arg Ala Leu Ile Arg Ser Leu Ala Lys Ser His Glu Val				
	625	630	635	640
ctg atc acg gtt gaa gaa ggt tcc att gga ggt ttt ggc tcg cac gtt				1968
Leu Ile Thr Val Glu Glu Gly Ser Ile Gly Gly Phe Gly Ser His Val				
	645	650	655	
gtt cag ttt ctt gct ctc gat ggt ctt ctt gat ggc aaa ctc aag tgg				2016
Val Gln Phe Leu Ala Leu Asp Gly Leu Leu Asp Gly Lys Leu Lys Trp				

WO 00/08169

PCT/EP99/05467

660	665	670	
aga cca atg gta ctg cct gat cga tac att gat cac ggt gca cca gct			2064
Arg Pro Met Val Leu Pro Asp Arg Tyr Ile Asp His Gly Ala Pro Ala			
675	680	685	
gat caa cta gct gaa gct gga ctc atg cca tct cac atc gca gca acc			2112
Asp Gln Leu Ala Glu Ala Gly Leu Met Pro Ser His Ile Ala Ala Thr			
690	695	700	
gca ctt aac tta atc ggt gca cca agg gaa gct ctg ttt tga			2154
Ala Leu Asn Leu Ile Gly Ala Pro Arg Glu Ala Leu Phe			
705	710	715	
gagtaagaat ctgttgcta aaacatatgt atacaaacac tctaaatgca acccaagggt 2214			
tcttctaagt actgatcaga attcccgccc gagaagtcct ttggcaacag ctatatatat 2274			
ttactaagat tgtgaagaga aaggcaaagg caaagggtgt gcaaagatta gtattataga 2334			
taaaactggt atttgttttg taattttagg attgtgatga gatcgtgttg taccaataac 2394			
taacatcttg taaaatcaat tactctcttg tgatcttcaa taagcttgag tgacaaaaaa 2454			
aaaa 2458			
<210> 2			
<211> 717			
<212> PRT			
<213> Arabidopsis thaliana			
<400> 2			
Met Ala Ser Ser Ala Phe Ala Phe Pro Ser Tyr Ile Ile Thr Lys Gly			
1	5	10	15
Gly Leu Ser Thr Asp Ser Cys Lys Ser Thr Ser Leu Ser Ser Ser Arg			
20	25	30	
Ser Leu Val Thr Asp Leu Pro Ser Pro Cys Leu Lys Pro Asn Asn Asn			
35	40	45	
Ser His Ser Asn Arg Arg Ala Lys Val Cys Ala Ser Leu Ala Glu Lys			
50	55	60	
Gly Glu Tyr Tyr Ser Asn Arg Pro Pro Thr Pro Leu Leu Asp Thr Ile			
65	70	75	80



WO 00/08169

PCT/EP99/05467

Asn Tyr Pro Ile His Met Lys Asn Leu Ser Val Lys Glu Leu Lys Gln  
                             85                            90                            95

Leu Ser Asp Glu Leu Arg Ser Asp Val Ile Phe Asn Val Ser Lys Thr  
                             100                            105                            110

Gly Gly His Leu Gly Ser Ser Leu Gly Val Val Glu Leu Thr Val Ala  
                             115                            120                            125

Leu His Tyr Ile Phe Asn Thr Pro Gln Asp Lys Ile Leu Trp Asp Val  
                             130                            135                            140

Gly His Gln Ser Tyr Pro His Lys Ile Leu Thr Gly Arg Arg Gly Lys  
 145                            150                            155                            160

Met Pro Thr Met Arg Gln Thr Asn Gly Leu Ser Gly Phe Thr Lys Arg  
                             165                            170                            175

Gly Glu Ser Glu His Asp Cys Phe Gly Thr Gly His Ser Ser Thr Thr  
                             180                            185                            190

Ile Ser Ala Gly Leu Gly Met Ala Val Gly Arg Asp Leu Lys Gly Lys  
                             195                            200                            205

Asn Asn Asn Val Val Ala Val Ile Gly Asp Gly Ala Met Thr Ala Gly  
                             210                            215                            220

Gln Ala Tyr Glu Ala Met Asn Asn Ala Gly Tyr Leu Asp Ser Asp Met  
 225                            230                            235                            240

Ile Val Ile Leu Asn Asp Asn Lys Gln Val Ser Leu Pro Thr Ala Thr  
                             245                            250                            255

Leu Asp Gly Pro Ser Pro Pro Val Gly Ala Leu Ser Ser Ala Leu Ser  
                             260                            265                            270

Arg Leu Gln Ser Asn Pro Ala Leu Arg Glu Leu Arg Glu Val Ala Lys  
                             275                            280                            285

Gly Met Thr Lys Gln Ile Gly Gly Pro Met His Gln Leu Ala Ala Lys  
                             290                            295                            300

Val Asp Val Tyr Ala Arg Gly Met Ile Ser Gly Thr Gly Ser Ser Leu  
 305                            310                            315                            320

Phe Glu Glu Leu Gly Leu Tyr Tyr Ile Gly Pro Val Asp Gly His Asn  
                             325                            330                            335

WO 00/08169

PCT/EP99/05467

Ile Asp Asp Leu Val Ala Ile Leu Lys Glu Val Lys Ser Thr Arg Thr  
 340 345 350

Thr Gly Pro Val Leu Ile His Val Val Thr Glu Lys Gly Arg Gly Tyr  
 355 360 365

Pro Tyr Ala Glu Arg Ala Asp Asp Lys Tyr His Gly Val Val Lys Phe  
 370 375 380

Asp Pro Ala Thr Gly Arg Gln Phe Lys Thr Thr Asn Glu Thr Gln Ser  
 385 390 395 400

Tyr Thr Thr Tyr Phe Ala Glu Ala Leu Val Ala Glu Ala Glu Val Asp  
 405 410 415

Lys Asp Val Val Ala Ile His Ala Ala Met Gly Gly Gly Thr Gly Leu  
 420 425 430

Asn Leu Phe Gln Arg Arg Phe Pro Thr Arg Cys Phe Asp Val Gly Ile  
 435 440 445

Ala Glu Gln His Ala Val Thr Phe Ala Ala Gly Leu Ala Cys Glu Gly  
 450 455 460

Leu Lys Pro Phe Cys Ala Ile Tyr Ser Ser Phe Met Gln Arg Ala Tyr  
 465 470 475 480

Asp Gln Val Val His Asp Val Asp Leu Gln Lys Leu Pro Val Arg Phe  
 485 490 495

Ala Met Asp Arg Ala Gly Leu Val Gly Ala Asp Gly Pro Thr His Cys  
 500 505 510

Gly Ala Phe Asp Val Thr Phe Met Ala Cys Leu Pro Asn Met Ile Val  
 515 520 525

Met Ala Pro Ser Asp Glu Ala Asp Leu Phe Asn Met Val Ala Thr Ala  
 530 535 540

Val Ala Ile Asp Asp Arg Pro Ser Cys Phe Arg Tyr Pro Arg Gly Asn  
 545 550 555 560

Gly Ile Gly Val Ala Leu Pro Pro Gly Asn Lys Gly Val Pro Ile Glu  
 565 570 575

Ile Gly Lys Gly Arg Ile Leu Lys Glu Gly Glu Arg Val Ala Leu Leu  
 580 585 590

WO 00/08169

PCT/EP99/05467

Gly Tyr Gly Ser Ala Val Gln Ser Cys Leu Gly Ala Ala Val Met Leu  
 595 600 605

Glu Glu Arg Gly Leu Asn Val Thr Val Ala Asp Ala Arg Phe Cys Lys  
 610 615 620

Pro Leu Asp Arg Ala Leu Ile Arg Ser Leu Ala Lys Ser His Glu Val  
 625 630 635 640

Leu Ile Thr Val Glu Glu Gly Ser Ile Gly Gly Phe Gly Ser His Val  
 645 650 655

Val Gln Phe Leu Ala Leu Asp Gly Leu Leu Asp Gly Lys Leu Lys Trp  
 660 665 670

Arg Pro Met Val Leu Pro Asp Arg Tyr Ile Asp His Gly Ala Pro Ala  
 675 680 685

Asp Gln Leu Ala Glu Ala Gly Leu Met Pro Ser His Ile Ala Ala Thr  
 690 695 700

Ala Leu Asn Leu Ile Gly Ala Pro Arg Glu Ala Leu Phe  
 705 710 715

&lt;210&gt; 3

&lt;211&gt; 1863

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1863)

&lt;400&gt; 3

atg agt ttt gat att gcc aaa tac ccg acc ctg gca ctg gtc gac tcc 48  
 Met Ser Phe Asp Ile Ala Lys Tyr Pro Thr Leu Ala Leu Val Asp Ser  
 1 5 10 15

acc cag gag tta cga ctg ttg ccg aaa gag agt tta ccg aaa ctc tgc 96  
 Thr Gln Glu Leu Arg Leu Leu Pro Lys Glu Ser Leu Pro Lys Leu Cys  
 20 25 30

gac gaa ctg cgc cgc tat tta ctc gac agc gtg agc cgt tcc agc ggg 144  
 Asp Glu Leu Arg Arg Tyr Leu Leu Asp Ser Val Ser Arg Ser Ser Gly  
 35 40 45

cac ttc gcc tcc ggg ctg ggc acg gtc gaa ctg acc gtg gcg ctg cac 192

WO 00/08169

PCT/EP99/05467

His Phe Ala Ser Gly Leu Gly Thr Val Glu Leu Thr Val Ala Leu His  
 50 55 60

tat gtc tac aac acc ccg ttt gac caa ttg att tgg gat gtg ggg cat 240  
 Tyr Val Tyr Asn Thr Pro Phe Asp Gln Leu Ile Trp Asp Val Gly His  
 65 70 75 80

cag gct tat ccg cat aaa att ttg acc gga cgc cgc gac aaa atc ggc 288  
 Gln Ala Tyr Pro His Lys Ile Leu Thr Gly Arg Arg Asp Lys Ile Gly  
 85 90 95

acc atc cgt cag aaa ggc ggt ctg cac ccg ttc ccg tgg cgc ggc gaa 336  
 Thr Ile Arg Gln Lys Gly Gly Leu His Pro Phe Pro Trp Arg Gly Glu  
 100 105 110

agc gaa tat gac gta tta agc gtc ggg cat tca tca acc tcc atc agt 384  
 Ser Glu Tyr Asp Val Leu Ser Val Gly His Ser Ser Thr Ser Ile Ser  
 115 120 125

gcc gga att ggt att gcg gtt gct gcc gaa aaa gaa ggc aaa aat cgc 432  
 Ala Gly Ile Gly Ile Ala Val Ala Ala Glu Lys Glu Gly Lys Asn Arg  
 130 135 140

cgc acc gtc tgt gtc att ggc gat ggc gcg att acc gca ggc atg gcg 480  
 Arg Thr Val Cys Val Ile Gly Asp Gly Ala Ile Thr Ala Gly Met Ala  
 145 150 155 160

ttt gaa gcg atg aat cac gcg ggc gat atc cgt cct gat atg ctg gtg 528  
 Phe Glu Ala Met Asn His Ala Gly Asp Ile Arg Pro Asp Met Leu Val  
 165 170 175

att ctc aac gac aat gaa atg tcg att tcc gaa aat gtc ggc gcg ctc 576  
 Ile Leu Asn Asp Asn Glu Met Ser Ile Ser Glu Asn Val Gly Ala Leu  
 180 185 190

aac aac cat ctg gca cag ctg ctt tcc ggt aag ctt tac tct tca ctg 624  
 Asn Asn His Leu Ala Gln Leu Leu Ser Gly Lys Leu Tyr Ser Ser Leu  
 195 200 205

cgc gaa ggc ggg aaa aaa gtt ttc tct ggc gtg ccg cca att aaa gag 672  
 Arg Glu Gly Gly Lys Lys Val Phe Ser Gly Val Pro Pro Ile Lys Glu  
 210 215 220

ctg ctc aaa cgc acc gaa gaa cat att aaa ggc atg gta gtg cct ggc 720  
 Leu Leu Lys Arg Thr Glu Glu His Ile Lys Gly Met Val Val Pro Gly  
 225 230 235 240

acg ttg ttt gaa gag ctg ggc ttt aac tac atc ggc ccg gtg gac ggt 768

WO 00/08169

PCT/EP99/05467

Thr Leu Phe Glu Glu Leu Gly Phe Asn Tyr Ile Gly Pro Val Asp Gly  
 245 250 255

cac gat gtg ctg ggg ctt atc acc acg cta aag aac atg cgc gac ctg 816  
 His Asp Val Leu Gly Leu Ile Thr Thr Leu Lys Asn Met Arg Asp Leu  
 260 265 270

aaa ggc ccg cag ttc ctg cat atc atg acc aaa aaa ggt cgt ggt tat 864  
 Lys Gly Pro Gln Phe Leu His Ile Met Thr Lys Lys Gly Arg Gly Tyr  
 275 280 285

gaa ccg gca gaa aaa gac ccg atc act ttc cac gcc gtg cct aaa ttt 912  
 Glu Pro Ala Glu Lys Asp Pro Ile Thr Phe His Ala Val Pro Lys Phe  
 290 295 300

gat ccc tcc agc ggt tgt ttg ccg aaa agt agc ggc ggt ttg ccg agc 960  
 Asp Pro Ser Ser Gly Cys Leu Pro Lys Ser Ser Gly Gly Leu Pro Ser  
 305 310 315 320

tat tca aaa atc ttt ggc gac tgg ttg tgc gaa acg gca gcg aaa gac 1008  
 Tyr Ser Lys Ile Phe Gly Asp Trp Leu Cys Glu Thr Ala Ala Lys Asp  
 325 330 335

aac aag ctg atg gcg att act ccg gcg atg cgt gaa ggt tcc ggc atg 1056  
 Asn Lys Leu Met Ala Ile Thr Pro Ala Met Arg Glu Gly Ser Gly Met  
 340 345 350

gtc gag ttt tca cgt aaa ttc ccg gat cgc tac ttc gac gtg gca att 1104  
 Val Glu Phe Ser Arg Lys Phe Pro Asp Arg Tyr Phe Asp Val Ala Ile  
 355 360 365

gcc gag caa cac gcg gtg acc ttt gct gcg ggt ctg gcg att ggt ggg 1152  
 Ala Glu Gln His Ala Val Thr Phe Ala Ala Gly Leu Ala Ile Gly Gly  
 370 375 380

tac aaa ccc att gtc gcg att tac tcc act ttc ctg caa cgc gcc tat 1200  
 Tyr Lys Pro Ile Val Ala Ile Tyr Ser Thr Phe Leu Gln Arg Ala Tyr  
 385 390 395 400

gat cag gtg ctg cat gac gtg gcg att caa aag ctt ccg gtc ctg ttc 1248  
 Asp Gln Val Leu His Asp Val Ala Ile Gln Lys Leu Pro Val Leu Phe  
 405 410 415

gcc atc gac cgc gcg ggc att gtt ggt gct gac ggt caa acc cat cag 1296  
 Ala Ile Asp Arg Ala Gly Ile Val Gly Ala Asp Gly Gln Thr His Gln  
 420 425 430

ggt gct ttt gat ctc tct tac ctg cgc tgc ata ccg gaa atg gtc att 1344

WO 00/08169

PCT/EP99/05467

Gly Ala Phe Asp Leu Ser Tyr Leu Arg Cys Ile Pro Glu Met Val Ile  
 435 440 445

atg acc ccg agc gat gaa aac gaa tgt cgc cag atg ctc tat acc ggc 1392  
 Met Thr Pro Ser Asp Glu Asn Glu Cys Arg Gln Met Leu Tyr Thr Gly  
 450 455 460

tat cac tat aac gat ggc ccg tca gcg gtg cgc tac ccg cgt ggc aac 1440  
 Tyr His Tyr Asn Asp Gly Pro Ser Ala Val Arg Tyr Pro Arg Gly Asn  
 465 470 475 480

gcg gtc ggc gtg gaa ctg acg ccg ctg gaa aaa cta cca att ggc aaa 1488  
 Ala Val Gly Val Glu Leu Thr Pro Leu Glu Lys Leu Pro Ile Gly Lys  
 485 490 495

ggc att gtg aag cgt cgt ggc gag aaa ctg gcg atc ctt aac ttt ggt 1536  
 Gly Ile Val Lys Arg Arg Gly Glu Lys Leu Ala Ile Leu Asn Phe Gly  
 500 505 510

acg ctg atg cca gaa gcg gcg aaa gtc gcc gaa tcg ctg aac gcc acg 1584  
 Thr Leu Met Pro Glu Ala Ala Lys Val Ala Glu Ser Leu Asn Ala Thr  
 515 520 525

ctg gtc gat atg cgt ttt gtg aaa ccg ctt gat gaa gcg tta att ctg 1632  
 Leu Val Asp Met Arg Phe Val Lys Pro Leu Asp Glu Ala Leu Ile Leu  
 530 535 540

gaa atg gcc gcc agc cat gaa gcg ctg gtc acc gta gaa gaa aac gcc 1680  
 Glu Met Ala Ala Ser His Glu Ala Leu Val Thr Val Glu Glu Asn Ala  
 545 550 555 560

att atg ggc ggc gca ggc agc ggc gtg aac gaa gtg ctg atg gcc cat 1728  
 Ile Met Gly Gly Ala Gly Ser Gly Val Asn Glu Val Leu Met Ala His  
 565 570 575

cgt aaa cca gta ccc gtg ctg aac att ggc ctg ccg gac ttc ttt att 1776  
 Arg Lys Pro Val Pro Val Leu Asn Ile Gly Leu Pro Asp Phe Phe Ile  
 580 585 590

ccg caa gga act cag gaa gaa atg cgc gcc gaa ctc ggc ctc gat gcc 1824  
 Pro Gln Gly Thr Gln Glu Glu Met Arg Ala Glu Leu Gly Leu Asp Ala  
 595 600 605

gct ggt atg gaa gcc aaa atc aag gcc tgg ctg gca taa 1863  
 Ala Gly Met Glu Ala Lys Ile Lys Ala Trp Leu Ala  
 610 615 620

WO 00/08169

PCT/EP99/05467

&lt;210&gt; 4

&lt;211&gt; 620

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 4

Met Ser Phe Asp Ile Ala Lys Tyr Pro Thr Leu Ala Leu Val Asp Ser  
 1 5 10 15

Thr Gln Glu Leu Arg Leu Leu Pro Lys Glu Ser Leu Pro Lys Leu Cys  
 20 25 30

Asp Glu Leu Arg Arg Tyr Leu Leu Asp Ser Val Ser Arg Ser Ser Gly  
 35 40 45

His Phe Ala Ser Gly Leu Gly Thr Val Glu Leu Thr Val Ala Leu His  
 50 55 60

Tyr Val Tyr Asn Thr Pro Phe Asp Gln Leu Ile Trp Asp Val Gly His  
 65 70 75 80

Gln Ala Tyr Pro His Lys Ile Leu Thr Gly Arg Arg Asp Lys Ile Gly  
 85 90 95

Thr Ile Arg Gln Lys Gly Gly Leu His Pro Phe Pro Trp Arg Gly Glu  
 100 105 110

Ser Glu Tyr Asp Val Leu Ser Val Gly His Ser Ser Thr Ser Ile Ser  
 115 120 125

Ala Gly Ile Gly Ile Ala Val Ala Ala Glu Lys Glu Gly Lys Asn Arg  
 130 135 140

Arg Thr Val Cys Val Ile Gly Asp Gly Ala Ile Thr Ala Gly Met Ala  
 145 150 155 160

Phe Glu Ala Met Asn His Ala Gly Asp Ile Arg Pro Asp Met Leu Val  
 165 170 175

Ile Leu Asn Asp Asn Glu Met Ser Ile Ser Glu Asn Val Gly Ala Leu  
 180 185 190

Asn Asn His Leu Ala Gln Leu Leu Ser Gly Lys Leu Tyr Ser Ser Leu  
 195 200 205

Arg Glu Gly Gly Lys Lys Val Phe Ser Gly Val Pro Pro Ile Lys Glu  
 210 215 220

WO 00/08169

PCT/EP99/05467

Leu Leu Lys Arg Thr Glu Glu His Ile Lys Gly Met Val Val Pro Gly  
 225 230 235 240

Thr Leu Phe Glu Glu Leu Gly Phe Asn Tyr Ile Gly Pro Val Asp Gly  
 245 250 255

His Asp Val Leu Gly Leu Ile Thr Thr Leu Lys Asn Met Arg Asp Leu  
 260 265 270

Lys Gly Pro Gln Phe Leu His Ile Met Thr Lys Lys Gly Arg Gly Tyr  
 275 280 285

Glu Pro Ala Glu Lys Asp Pro Ile Thr Phe His Ala Val Pro Lys Phe  
 290 295 300

Asp Pro Ser Ser Gly Cys Leu Pro Lys Ser Ser Gly Gly Leu Pro Ser  
 305 310 315 320

Tyr Ser Lys Ile Phe Gly Asp Trp Leu Cys Glu Thr Ala Ala Lys Asp  
 325 330 335

Asn Lys Leu Met Ala Ile Thr Pro Ala Met Arg Glu Gly Ser Gly Met  
 340 345 350

Val Glu Phe Ser Arg Lys Phe Pro Asp Arg Tyr Phe Asp Val Ala Ile  
 355 360 365

Ala Glu Gln His Ala Val Thr Phe Ala Ala Gly Leu Ala Ile Gly Gly  
 370 375 380

Tyr Lys Pro Ile Val Ala Ile Tyr Ser Thr Phe Leu Gln Arg Ala Tyr  
 385 390 395 400

Asp Gln Val Leu His Asp Val Ala Ile Gln Lys Leu Pro Val Leu Phe  
 405 410 415

Ala Ile Asp Arg Ala Gly Ile Val Gly Ala Asp Gly Gln Thr His Gln  
 420 425 430

Gly Ala Phe Asp Leu Ser Tyr Leu Arg Cys Ile Pro Glu Met Val Ile  
 435 440 445

Met Thr Pro Ser Asp Glu Asn Glu Cys Arg Gln Met Leu Tyr Thr Gly  
 450 455 460

Tyr His Tyr Asn Asp Gly Pro Ser Ala Val Arg Tyr Pro Arg Gly Asn  
 465 470 475 480



WO 00/08169

PCT/EP99/05467

Ala Val Gly Val Glu Leu Thr Pro Leu Glu Lys Leu Pro Ile Gly Lys  
                     485                    490                    495

Gly Ile Val Lys Arg Arg Gly Glu Lys Leu Ala Ile Leu Asn Phe Gly  
                     500                    505                    510

Thr Leu Met Pro Glu Ala Ala Lys Val Ala Glu Ser Leu Asn Ala Thr  
                     515                    520                    525

Leu Val Asp Met Arg Phe Val Lys Pro Leu Asp Glu Ala Leu Ile Leu  
                     530                    535                    540

Glu Met Ala Ala Ser His Glu Ala Leu Val Thr Val Glu Glu Asn Ala  
                     545                    550                    555                    560

Ile Met Gly Gly Ala Gly Ser Gly Val Asn Glu Val Leu Met Ala His  
                     565                    570                    575

Arg Lys Pro Val Pro Val Leu Asn Ile Gly Leu Pro Asp Phe Phe Ile  
                     580                    585                    590

Pro Gln Gly Thr Gln Glu Glu Met Arg Ala Glu Leu Gly Leu Asp Ala  
                     595                    600                    605

Ala Gly Met Glu Ala Lys Ile Lys Ala Trp Leu Ala  
                     610                    615                    620

&lt;210&gt; 5

&lt;211&gt; 1469

&lt;212&gt; DNA

&lt;213&gt; Streptomyces avermitilis

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (218) .. (1138)

&lt;400&gt; 5

gatatccgag cgccgccggg tccactgagg tccgaagccg cggatgactc cattcgactg 60

aagccggtcg agccgcgcct gcacgggtgcc ggcgcgcgacc ccgagccgcc gggacatctc 120

gagcactccg atgcgcgggt cccgcgccag cagcaccagg agccggccgt ccagatgata 180

gatgccacg gcagcccctc cagtggatcat cctgtac atg cag ccc cac gcc atg 235

Met Gln Pro His Ala Met

1

5

WO 00/08169

PCT/EP99/05467

ggc ggt gca ctg aac aca ttg tcc agc gga caa gcc aac tat tgc gca 283  
 Gly Gly Ala Leu Asn Thr Leu Ser Ser Gly Gln Ala Asn Tyr Cys Ala  
 10 15 20

cct tgc gga acg gag cga ccc tgc cgc cat gac gca gac cac aca cca 331  
 Pro Cys Gly Thr Glu Arg Pro Cys Arg His Asp Ala Asp His Thr Pro  
 25 30 35

cac tcc cga cac cgc ccg gca ggc cga ccc ctt ccc ggt gaa ggg aat 379  
 His Ser Arg His Arg Pro Ala Gly Arg Pro Leu Pro Gly Glu Gly Asn  
 40 45 50

gga cgc ggt cgt ctt cgc cgt agg caa cgc caa gca ggc cgc gca cta 427  
 Gly Arg Gly Arg Leu Arg Arg Arg Gln Arg Gln Ala Gly Arg Ala Leu  
 55 60 65 70

ctc cac cgc ctt cgg cat gca gct tgt ggc gta ctc cgg acc gga gaa 475  
 Leu His Arg Leu Arg His Ala Ala Cys Gly Val Leu Arg Thr Gly Glu  
 75 80 85

cgg cag ccg cga gac cgc ttc gta cgt cct cac caa cgg ctc ggc acg 523  
 Arg Gln Pro Arg Asp Arg Phe Val Arg Pro His Gln Arg Leu Gly Thr  
 90 95 100

ctt cgt cct cac ctc cgt cat caa gcc cgc cac ccc ctg ggg cca ctt 571  
 Leu Arg Pro His Leu Arg His Gln Ala Arg His Pro Leu Gly Pro Leu  
 105 110 115

cct cgc cga cca tgt ggc cga gca cgg cga cgg cgt cgt cga cct cgc 619  
 Pro Arg Arg Pro Cys Gly Arg Ala Arg Arg Arg Arg Arg Pro Arg  
 120 125 130

cat cga ggt ccc gga cgc ccg cgc cgc cca cgc gta cgc gat cga gca 667  
 His Arg Gly Pro Gly Arg Pro Arg Arg Pro Arg Val Arg Asp Arg Ala  
 135 140 145 150

cgg cgc ccg ctc ggt cgc cga gcc gta cga gct gaa gga cga gca cgg 715  
 Arg Arg Pro Leu Gly Arg Arg Ala Val Arg Ala Glu Gly Arg Ala Arg  
 155 160 165

cac ggt cgt cct cgc cgc gat cgc cac cta cgg caa gac ccg cca cac 763  
 His Gly Arg Pro Arg Arg Asp Arg His Leu Arg Gln Asp Pro Pro His  
 170 175 180

cct cgt cga ccg gac cgg cta cga cgg ccc cta cct ccc cgg cta cgt 811  
 Pro Arg Arg Pro Asp Arg Leu Arg Arg Pro Leu Pro Pro Arg Leu Arg  
 185 190 195

WO 00/08169

PCT/EP99/05467

ggc cgc cgc ccc gat cgt cga acc gcc cgc cca ccg cac ctt cca ggc 859  
 Gly Arg Arg Pro Asp Arg Arg Thr Ala Arg Pro Pro His Leu Pro Gly  
 200 205 210

cat cga cca ctg cgt cgg caa cgt cga gct cgg ccg gat gaa cga atg 907  
 His Arg Pro Leu Arg Arg Gln Arg Arg Ala Arg Pro Asp Glu Arg Met  
 215 220 225 230

ggt cgg ctt cta caa caa ggt cat ggg ctt cac gaa cat gaa gga gtt 955  
 Gly Arg Leu Leu Gln Gln Gly His Gly Leu His Glu His Glu Gly Val  
 235 240 245

cgt ggg cga cga cat cgc gac cga gta ctc ggc gct gat gtc gaa ggt 1003  
 Arg Gly Arg Arg His Arg Asp Arg Val Leu Gly Ala Asp Val Glu Gly  
 250 255 260

cgt ggc cga cgg cac gct caa ggt caa gtt ccc gat caa cga gcc cgc 1051  
 Arg Gly Arg Arg His Ala Gln Gly Gln Val Pro Asp Gln Arg Ala Arg  
 265 270 275

cct cgc caa gaa gaa gtc cca gat cga cga gta cct gga gtt cta cgg 1099  
 Pro Arg Gln Glu Glu Val Pro Asp Arg Arg Val Pro Gly Val Leu Arg  
 280 285 290

cgg cgc ggg cgt cca gca cat cgc gct gaa cac ggg tga catcgtcgag 1148  
 Arg Arg Gly Arg Pro Ala His Arg Ala Glu His Gly  
 295 300 305

acggtacgca cgatgcgcgc cgccggcgctc cagttcctgg acacgcccga ctcgtactac 1208  
 gacaccctcg gggagtgggt gggcgacacc cgcgtcccg tcgacaccct gcgcgagctg 1268  
 aagatcctcg cggaccgcga cgaggacggc tatctgctcc agatcttcac caagccggtc 1328  
 caggaccgcc cgacggtctt cttcgagatc atcgaacgcc acggctcgat gggattcggc 1388  
 aagggcaact tcaaggccct gttcgaggcg atcgagcggg agcaggagaa gcggggcaac 1448  
 ctgtaggcgg cgcgcccg g 1469

<210> 6  
 <211> 306  
 <212> PRT  
 <213> Streptomyces avermitilis

<400> 6  
 Met Gln Pro His Ala Met Gly Gly Ala Leu Asn Thr Leu Ser Ser Gly

WO 00/08169

PCT/EP99/05467

1                      5                      10                      15  
 Gln Ala Asn Tyr Cys Ala Pro Cys Gly Thr Glu Arg Pro Cys Arg His  
                     20                      25                      30  
 Asp Ala Asp His Thr Pro His Ser Arg His Arg Pro Ala Gly Arg Pro  
                     35                      40                      45  
 Leu Pro Gly Glu Gly Asn Gly Arg Gly Arg Leu Arg Arg Arg Gln Arg  
                     50                      55                      60  
 Gln Ala Gly Arg Ala Leu Leu His Arg Leu Arg His Ala Ala Cys Gly  
                     65                      70                      75                      80  
 Val Leu Arg Thr Gly Glu Arg Gln Pro Arg Asp Arg Phe Val Arg Pro  
                     85                      90                      95  
 His Gln Arg Leu Gly Thr Leu Arg Pro His Leu Arg His Gln Ala Arg  
                     100                      105                      110  
 His Pro Leu Gly Pro Leu Pro Arg Arg Pro Cys Gly Arg Ala Arg Arg  
                     115                      120                      125  
 Arg Arg Arg Arg Pro Arg His Arg Gly Pro Gly Arg Pro Arg Arg Pro  
                     130                      135                      140  
 Arg Val Arg Asp Arg Ala Arg Arg Pro Leu Gly Arg Arg Ala Val Arg  
                     145                      150                      155                      160  
 Ala Glu Gly Arg Ala Arg His Gly Arg Pro Arg Arg Asp Arg His Leu  
                     165                      170                      175  
 Arg Gln Asp Pro Pro His Pro Arg Arg Pro Asp Arg Leu Arg Arg Pro  
                     180                      185                      190  
 Leu Pro Pro Arg Leu Arg Gly Arg Arg Pro Asp Arg Arg Thr Ala Arg  
                     195                      200                      205  
 Pro Pro His Leu Pro Gly His Arg Pro Leu Arg Arg Gln Arg Arg Ala  
                     210                      215                      220  
 Arg Pro Asp Glu Arg Met Gly Arg Leu Leu Gln Gln Gly His Gly Leu  
                     225                      230                      235                      240  
 His Glu His Glu Gly Val Arg Gly Arg Arg His Arg Asp Arg Val Leu  
                     245                      250                      255  
 Gly Ala Asp Val Glu Gly Arg Gly Arg Arg His Ala Gln Gly Gln Val

WO 00/08169

PCT/EP99/05467

260 265 270  
 Pro Asp Gln Arg Ala Arg Pro Arg Gln Glu Glu Val Pro Asp Arg Arg  
 275 280 285  
 Val Pro Gly Val Leu Arg Arg Arg Gly Arg Pro Ala His Arg Ala Glu  
 290 295 300  
 His Gly  
 305  
  
 <210> 7  
 <211> 1479  
 <212> DNA  
 <213> Arabidopsis thaliana  
  
 <220>  
 <221> CDS  
 <222> (1)..(1401)  
  
 <400> 7  
 atg gcg acg acg gtt aca ctc aaa tcc ttc acc gga ctt cgt caa tca 48  
 Met Ala Thr Thr Val Thr Leu Lys Ser Phe Thr Gly Leu Arg Gln Ser  
 1 5 10 15  
 tca acg gag caa aca aac ttc gtc tct cat gta ccg tca tca ctt tct 96  
 Ser Thr Glu Gln Thr Asn Phe Val Ser His Val Pro Ser Ser Leu Ser  
 20 25 30  
 ctc cct caa cga cgg acc tct ctc cga gta acc gca gcc agg gcc act 144  
 Leu Pro Gln Arg Arg Thr Ser Leu Arg Val Thr Ala Ala Arg Ala Thr  
 35 40 45  
 ccc aaa ctc tcc aac cgt aaa ctc cgt gtc gcc gtc atc ggt ggt gga 192  
 Pro Lys Leu Ser Asn Arg Lys Leu Arg Val Ala Val Ile Gly Gly Gly  
 50 55 60  
 cca gca ggc ggg gca gct gca gag act cta gca caa gga gga atc gag 240  
 Pro Ala Gly Gly Ala Ala Ala Glu Thr Leu Ala Gln Gly Gly Ile Glu  
 65 70 75 80  
 acg att ctc atc gag cgt aag atg gac aat tgc aag cct tgc ggt ggc 288  
 Thr Ile Leu Ile Glu Arg Lys Met Asp Asn Cys Lys Pro Cys Gly Gly  
 85 90 95  
 gcg att cct ctc tgt atg gtc gga gaa ttc aac ttg ccg ttg gat att 336  
 Ala Ile Pro Leu Cys Met Val Gly Glu Phe Asn Leu Pro Leu Asp Ile

WO 00/08169

PCT/EP99/05467

100	105	110	
att gat cgg aga gtg acg aag atg aag atg att tcg ccg tcg aac att			384
Ile Asp Arg Arg Val Thr Lys Met Lys Met Ile Ser Pro Ser Asn Ile			
115	120	125	
gct gtt gat att ggt cgt acg ctt aag gag cat gag tat ata ggt atg			432
Ala Val Asp Ile Gly Arg Thr Leu Lys Glu His Glu Tyr Ile Gly Met			
130	135	140	
gtg aga aga gaa gtt ctt gat gct tat ctg aga gag aga gct gag aag			480
Val Arg Arg Glu Val Leu Asp Ala Tyr Leu Arg Glu Arg Ala Glu Lys			
145	150	155	160
agt gga gcc act gtg att aac ggt ctc ttc ctt aag atg gat cat ccg			528
Ser Gly Ala Thr Val Ile Asn Gly Leu Phe Leu Lys Met Asp His Pro			
165	170	175	
gag aat tgg gac tcg ccg tac act ttg cat tac act gag tac gat ggt			576
Glu Asn Trp Asp Ser Pro Tyr Thr Leu His Tyr Thr Glu Tyr Asp Gly			
180	185	190	
aaa act gga gct aca ggg acg aag aaa aca atg gag gtt gat gct gtc			624
Lys Thr Gly Ala Thr Gly Thr Lys Lys Thr Met Glu Val Asp Ala Val			
195	200	205	
att gga gct gat gga gct aac tct agg gtt gct aaa tct att gat gct			672
Ile Gly Ala Asp Gly Ala Asn Ser Arg Val Ala Lys Ser Ile Asp Ala			
210	215	220	
ggt gat tac gac tac gca att gca ttt cag gag agg att agg att cct			720
Gly Asp Tyr Asp Tyr Ala Ile Ala Phe Gln Glu Arg Ile Arg Ile Pro			
225	230	235	240
gat gag aaa atg act tac tat gag gat tta gct gag atg tat gtt gga			768
Asp Glu Lys Met Thr Tyr Tyr Glu Asp Leu Ala Glu Met Tyr Val Gly			
245	250	255	
gat gat gtg tcg ccg gat ttc tat ggt tgg gtg ttc cct aag tgc gac			816
Asp Asp Val Ser Pro Asp Phe Tyr Gly Trp Val Phe Pro Lys Cys Asp			
260	265	270	
cat gta gct gtt gga aca ggt act gtg act cac aaa ggt gac atc aag			864
His Val Ala Val Gly Thr Gly Thr Val Thr His Lys Gly Asp Ile Lys			
275	280	285	
aag ttc cag ctc gcg acc aga aac aga gct aag gac aag att ctt gga			912
Lys Phe Gln Leu Ala Thr Arg Asn Arg Ala Lys Asp Lys Ile Leu Gly			

WO 00/08169

PCT/EP99/05467

290	295	300	
ggg aag atc atc cgt gtg gag gct cat ccg att cct gaa cat ccg aga			960
Gly Lys Ile Ile Arg Val Glu Ala His Pro Ile Pro Glu His Pro Arg			
305	310	315	320
cca cgt agg ctc tcg aaa cgt gtg gct ctt gta ggt gat gct gca ggg			1008
Pro Arg Arg Leu Ser Lys Arg Val Ala Leu Val Gly Asp Ala Ala Gly			
325	330	335	
tat gtg act aaa tgc tct ggt gaa ggg atc tac ttt gct gct aag agt			1056
Tyr Val Thr Lys Cys Ser Gly Glu Gly Ile Tyr Phe Ala Ala Lys Ser			
340	345	350	
gga aga atg tgt gct gaa gcc att gtc gaa ggt tca cag aat ggt aag			1104
Gly Arg Met Cys Ala Glu Ala Ile Val Glu Gly Ser Gln Asn Gly Lys			
355	360	365	
aag atg att gac gaa ggg gac ttg agg aag tac ttg gag aaa tgg gat			1152
Lys Met Ile Asp Glu Gly Asp Leu Arg Lys Tyr Leu Glu Lys Trp Asp			
370	375	380	
aag aca tac ttg cct acc tac agg gta ctt gat gtg ttg cag aaa gtg			1200
Lys Thr Tyr Leu Pro Thr Tyr Arg Val Leu Asp Val Leu Gln Lys Val			
385	390	395	400
ttt tac aga tca aat ccg gct aga gaa gcg ttt gtg gag atg tgt aat			1248
Phe Tyr Arg Ser Asn Pro Ala Arg Glu Ala Phe Val Glu Met Cys Asn			
405	410	415	
gat gag tat gtt cag aag atg aca ttc gat agc tat ctg tac aag cgg			1296
Asp Glu Tyr Val Gln Lys Met Thr Phe Asp Ser Tyr Leu Tyr Lys Arg			
420	425	430	
gtt gcg ccg ggt agt cct ttg gag gat atc aag ttg gct gtg aac acc			1344
Val Ala Pro Gly Ser Pro Leu Glu Asp Ile Lys Leu Ala Val Asn Thr			
435	440	445	
att gga agt ttg gtt agg gct aat gct cta agg aga gag att gag aag			1392
Ile Gly Ser Leu Val Arg Ala Asn Ala Leu Arg Arg Glu Ile Glu Lys			
450	455	460	
ctt agt gtt taagaaacaa ataatgaggt ctatctcctt tcttcatctc			1441
Leu Ser Val			
465			
tatctctctt tttttgtctg ttagtaatct atctacac			1479

WO 00/08169

PCT/EP99/05467

&lt;210&gt; 8

&lt;211&gt; 467

&lt;212&gt; PRT

&lt;213&gt; Arabidopsis thaliana

&lt;400&gt; 8

Met Ala Thr Thr Val Thr Leu Lys Ser Phe Thr Gly Leu Arg Gln Ser  
 1 5 10 15

Ser Thr Glu Gln Thr Asn Phe Val Ser His Val Pro Ser Ser Leu Ser  
 20 25 30

Leu Pro Gln Arg Arg Thr Ser Leu Arg Val Thr Ala Ala Arg Ala Thr  
 35 40 45

Pro Lys Leu Ser Asn Arg Lys Leu Arg Val Ala Val Ile Gly Gly Gly  
 50 55 60

Pro Ala Gly Gly Ala Ala Ala Glu Thr Leu Ala Gln Gly Gly Ile Glu  
 65 70 75 80

Thr Ile Leu Ile Glu Arg Lys Met Asp Asn Cys Lys Pro Cys Gly Gly  
 85 90 95

Ala Ile Pro Leu Cys Met Val Gly Glu Phe Asn Leu Pro Leu Asp Ile  
 100 105 110

Ile Asp Arg Arg Val Thr Lys Met Lys Met Ile Ser Pro Ser Asn Ile  
 115 120 125

Ala Val Asp Ile Gly Arg Thr Leu Lys Glu His Glu Tyr Ile Gly Met  
 130 135 140

Val Arg Arg Glu Val Leu Asp Ala Tyr Leu Arg Glu Arg Ala Glu Lys  
 145 150 155 160

Ser Gly Ala Thr Val Ile Asn Gly Leu Phe Leu Lys Met Asp His Pro  
 165 170 175

Glu Asn Trp Asp Ser Pro Tyr Thr Leu His Tyr Thr Glu Tyr Asp Gly  
 180 185 190

Lys Thr Gly Ala Thr Gly Thr Lys Lys Thr Met Glu Val Asp Ala Val  
 195 200 205

Ile Gly Ala Asp Gly Ala Asn Ser Arg Val Ala Lys Ser Ile Asp Ala  
 210 215 220



WO 00/08169

PCT/EP99/05467

Gly Asp Tyr Asp Tyr Ala Ile Ala Phe Gln Glu Arg Ile Arg Ile Pro  
 225 230 235 240

Asp Glu Lys Met Thr Tyr Tyr Glu Asp Leu Ala Glu Met Tyr Val Gly  
 245 250 255

Asp Asp Val Ser Pro Asp Phe Tyr Gly Trp Val Phe Pro Lys Cys Asp  
 260 265 270

His Val Ala Val Gly Thr Gly Thr Val Thr His Lys Gly Asp Ile Lys  
 275 280 285

Lys Phe Gln Leu Ala Thr Arg Asn Arg Ala Lys Asp Lys Ile Leu Gly  
 290 295 300

Gly Lys Ile Ile Arg Val Glu Ala His Pro Ile Pro Glu His Pro Arg  
 305 310 315 320

Pro Arg Arg Leu Ser Lys Arg Val Ala Leu Val Gly Asp Ala Ala Gly  
 325 330 335

Tyr Val Thr Lys Cys Ser Gly Glu Gly Ile Tyr Phe Ala Ala Lys Ser  
 340 345 350

Gly Arg Met Cys Ala Glu Ala Ile Val Glu Gly Ser Gln Asn Gly Lys  
 355 360 365

Lys Met Ile Asp Glu Gly Asp Leu Arg Lys Tyr Leu Glu Lys Trp Asp  
 370 375 380

Lys Thr Tyr Leu Pro Thr Tyr Arg Val Leu Asp Val Leu Gln Lys Val  
 385 390 395 400

Phe Tyr Arg Ser Asn Pro Ala Arg Glu Ala Phe Val Glu Met Cys Asn  
 405 410 415

Asp Glu Tyr Val Gln Lys Met Thr Phe Asp Ser Tyr Leu Tyr Lys Arg  
 420 425 430

Val Ala Pro Gly Ser Pro Leu Glu Asp Ile Lys Leu Ala Val Asn Thr  
 435 440 445

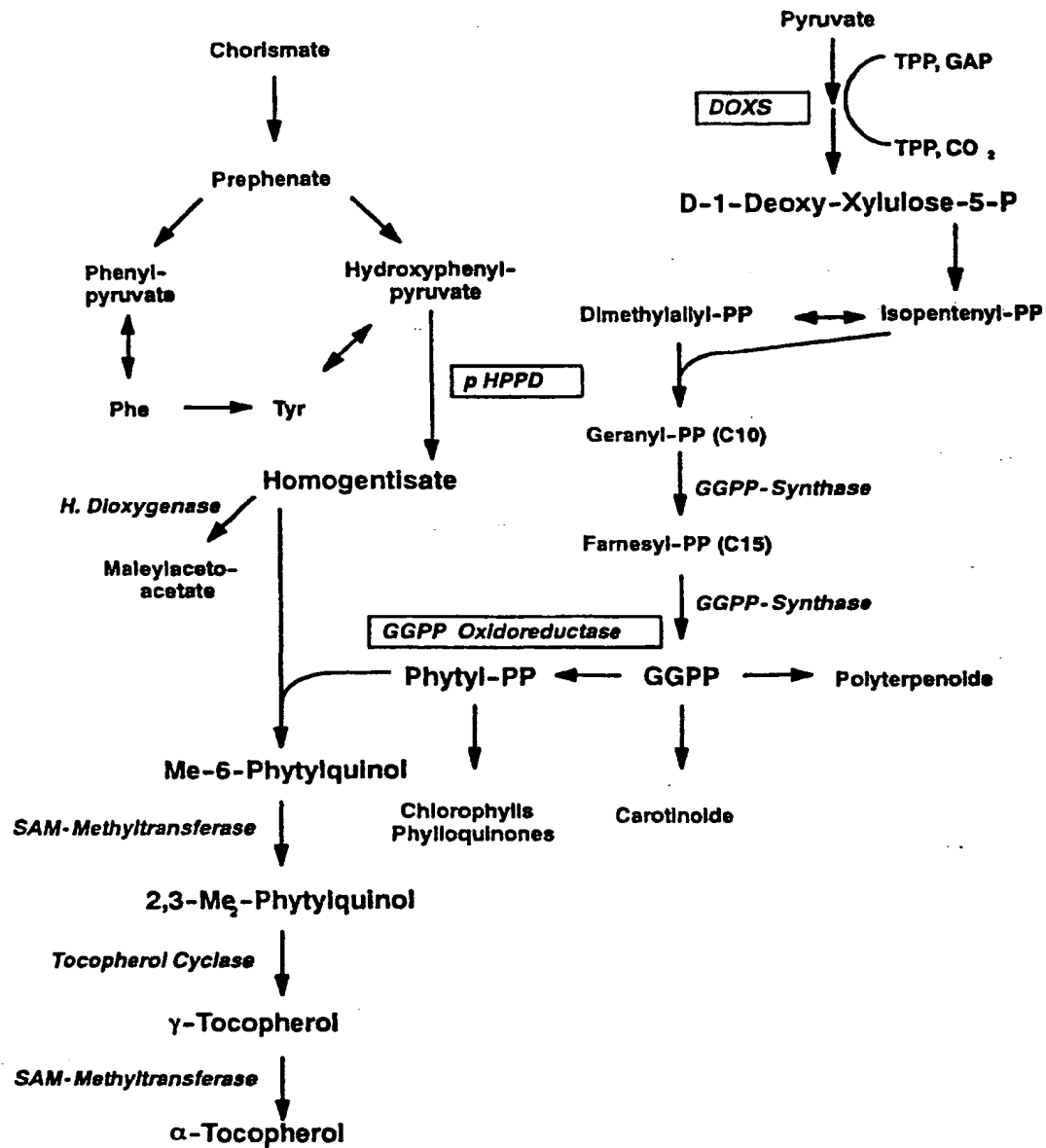
Ile Gly Ser Leu Val Arg Ala Asn Ala Leu Arg Arg Glu Ile Glu Lys  
 450 455 460

Leu Ser Val  
 465

0817/00006

1/NO TAG

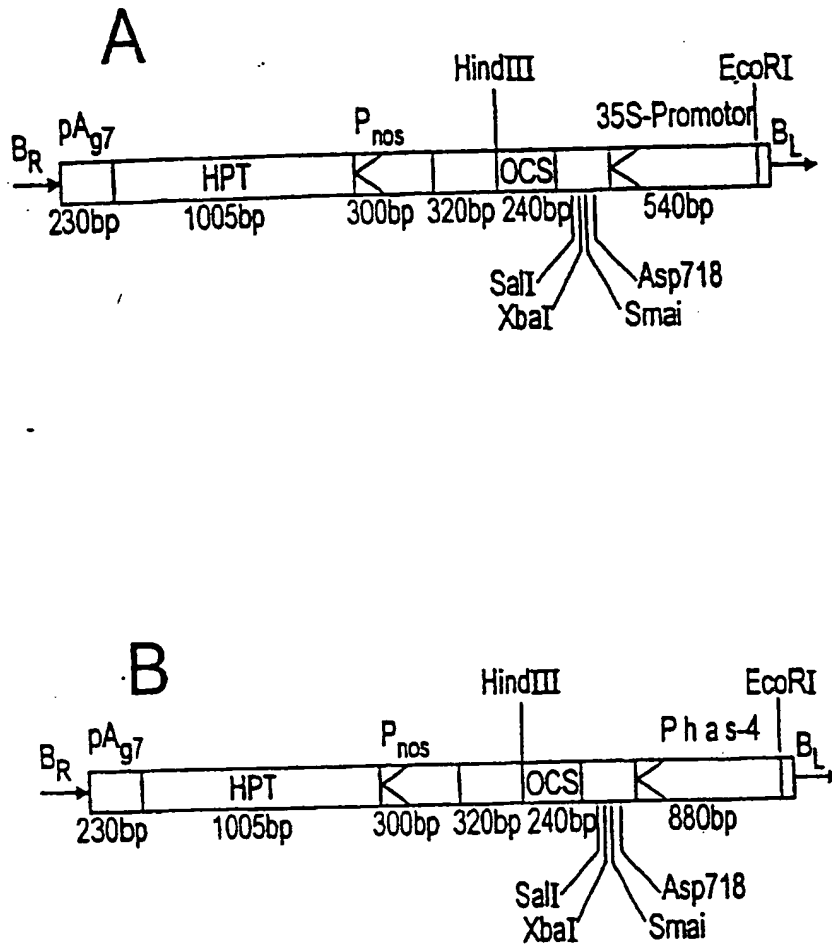
Figure 1



0817/00006

2/NO TAG

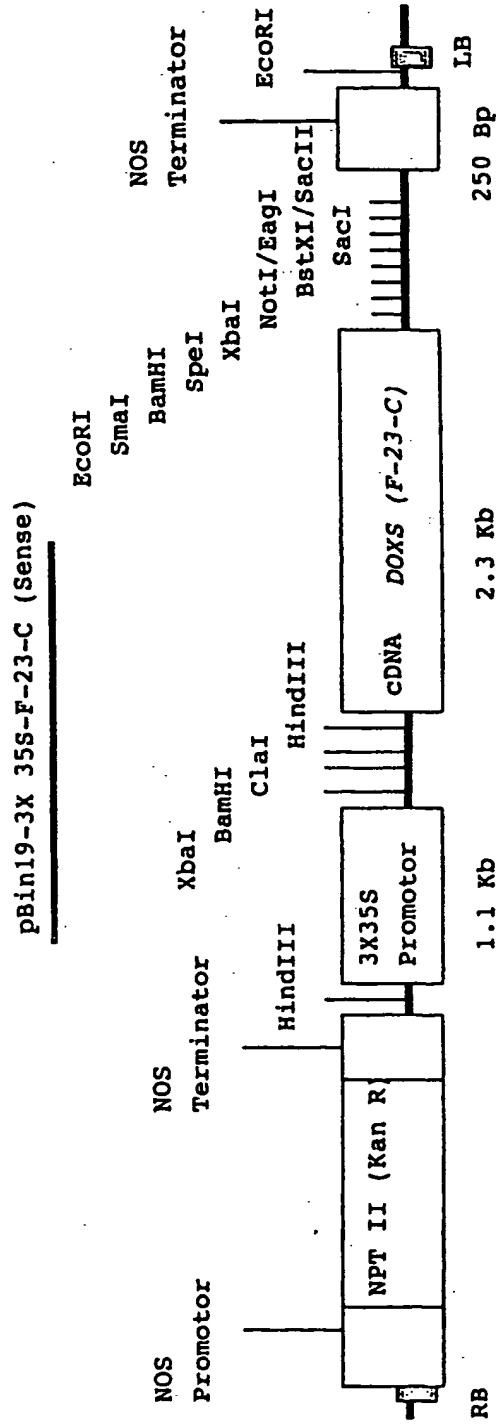
Figure 2



0817/00006

3/11

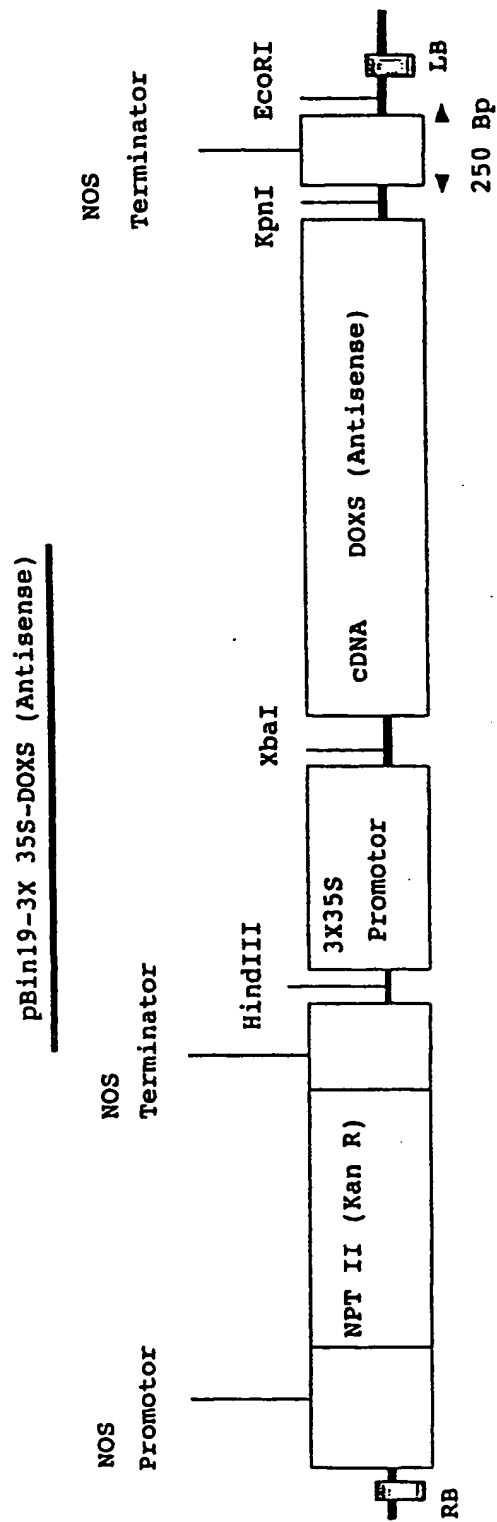
Figure 3



0817/00006

4/11

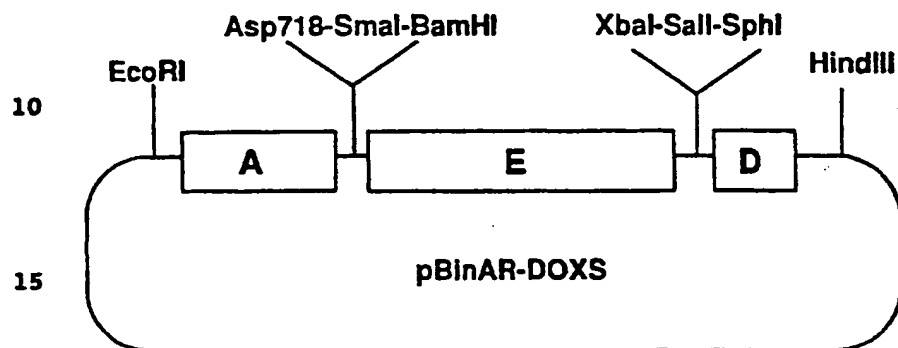
Figure 4



1/NO TAG

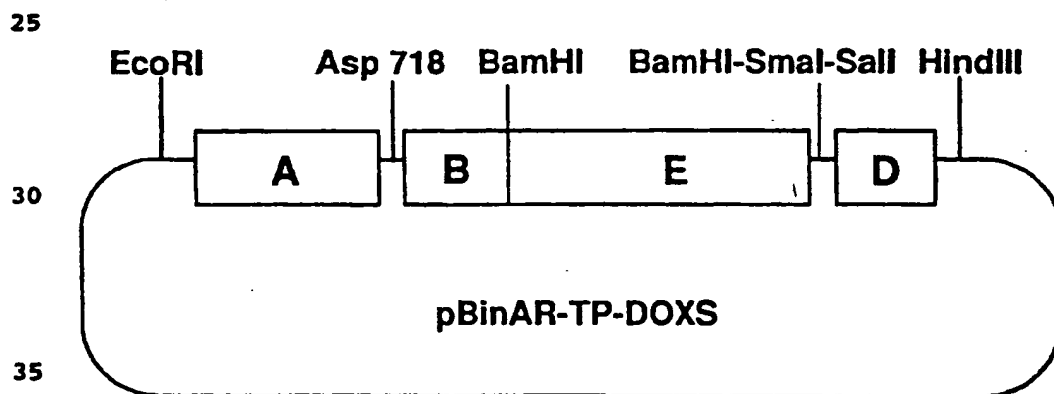
Figure 5

Binary vector for overexpression of the DOXS gene from E. coli in the cytosol of transgenic plants



20 Figure 6

Binary vector for overexpression of the DOXS gene from E.coli in plastids of transgenic plants.



## 2/NO TAG

Figure 7: DOXS gene RNA expression level

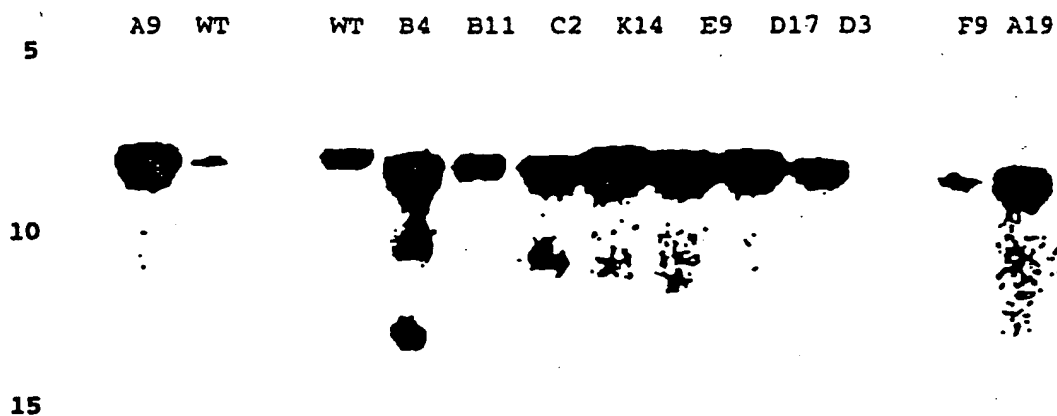
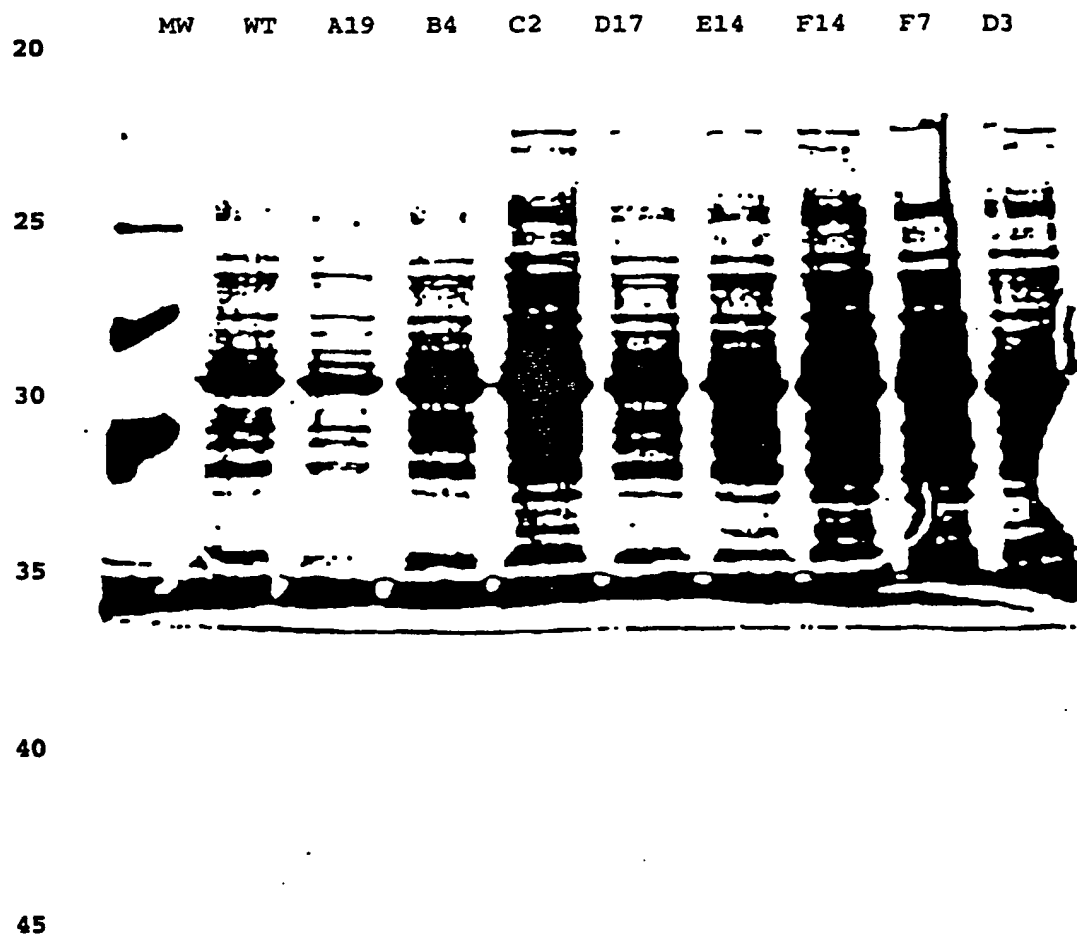


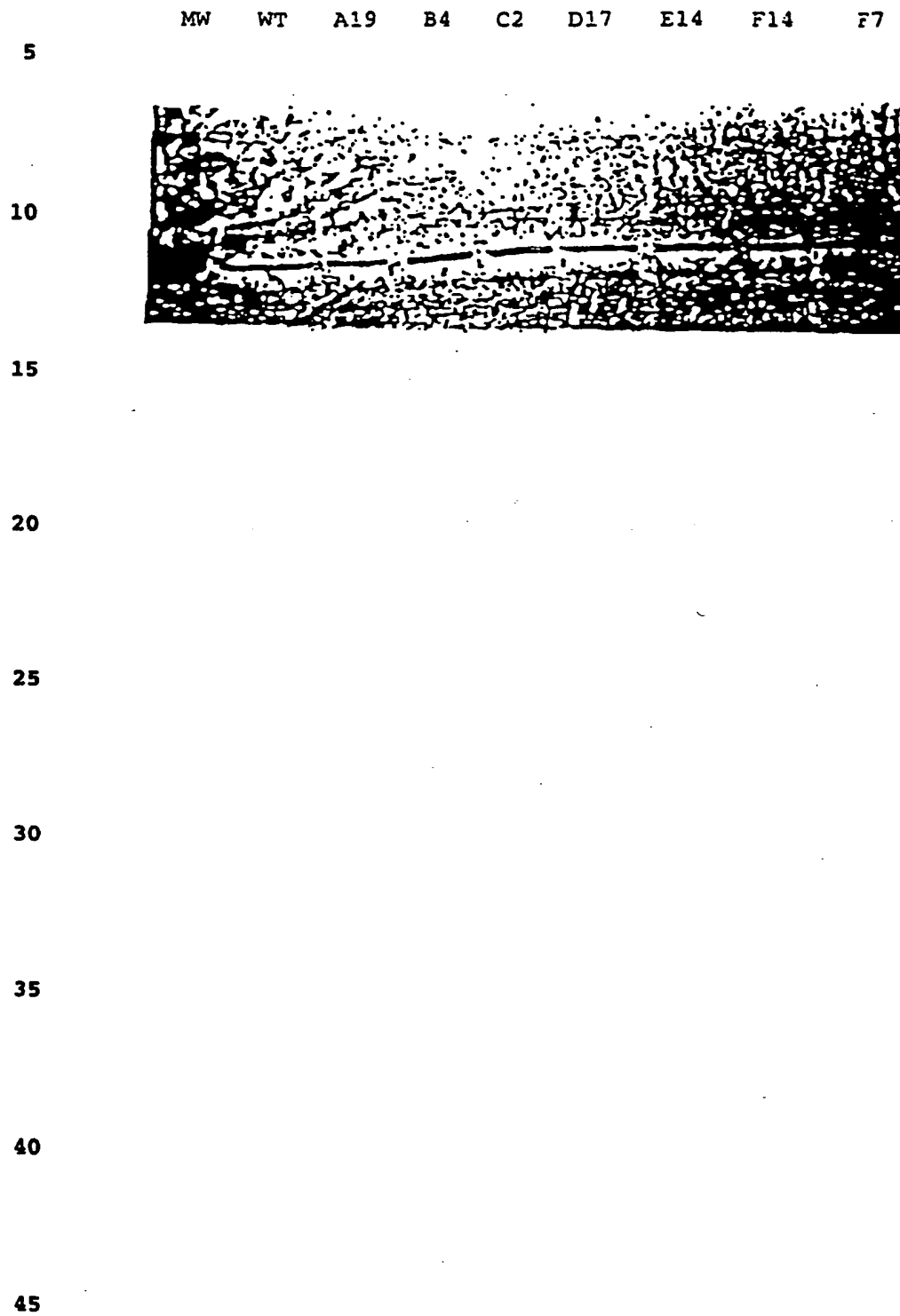
Figure 8: Amounts of protein in transgenic plants



0817/00006

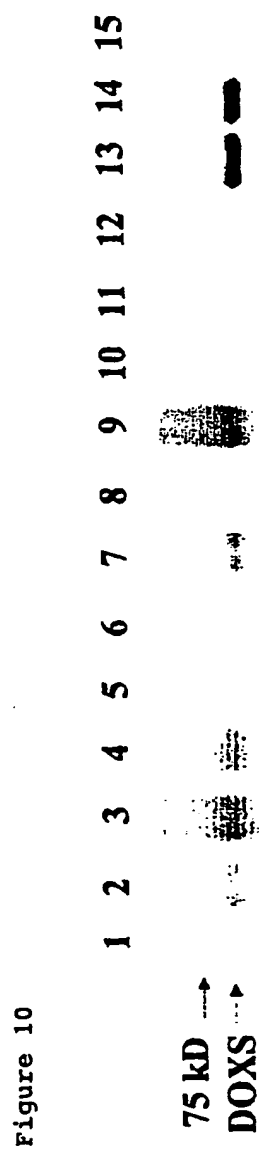
3/NO TAG

Figure 9: Western analysis





0817/00006

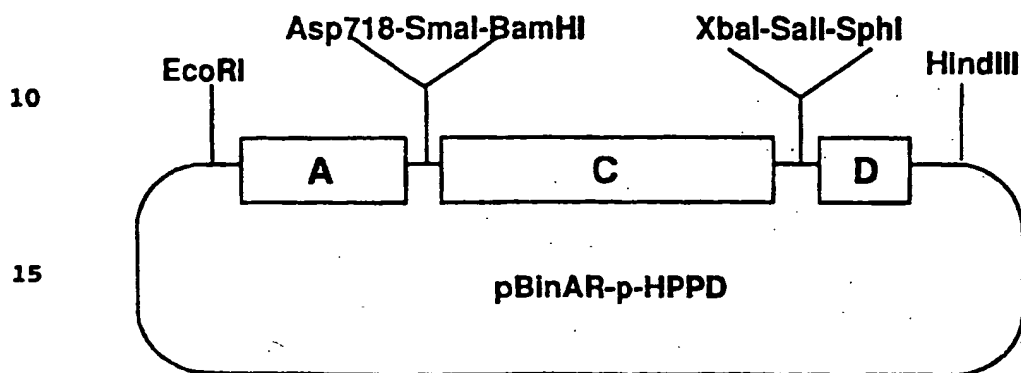


0817/00006

1

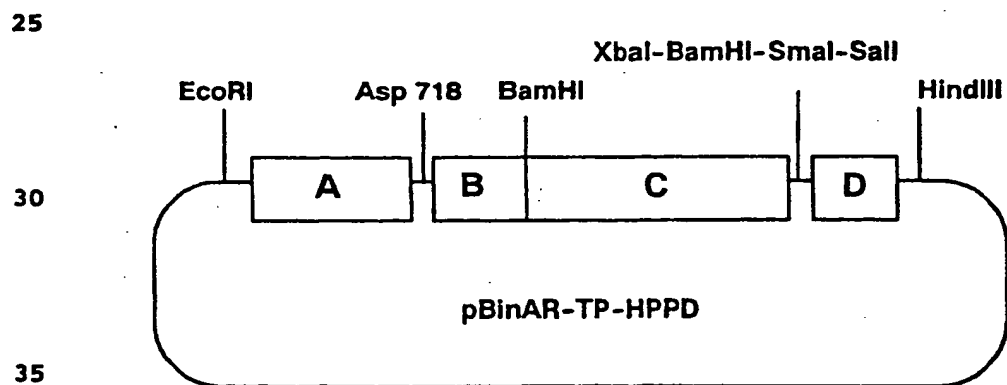
Figure 11

Binary vector for overexpression of the HPPD gene from  
5 *Streptomyces avermitilis* in the cytosol of transgenic plants



20 Figure 12

Binary vector for overexpression of the HPPD gene from  
25 *Streptomyces avermitilis* in plastids of transgenic plants



40

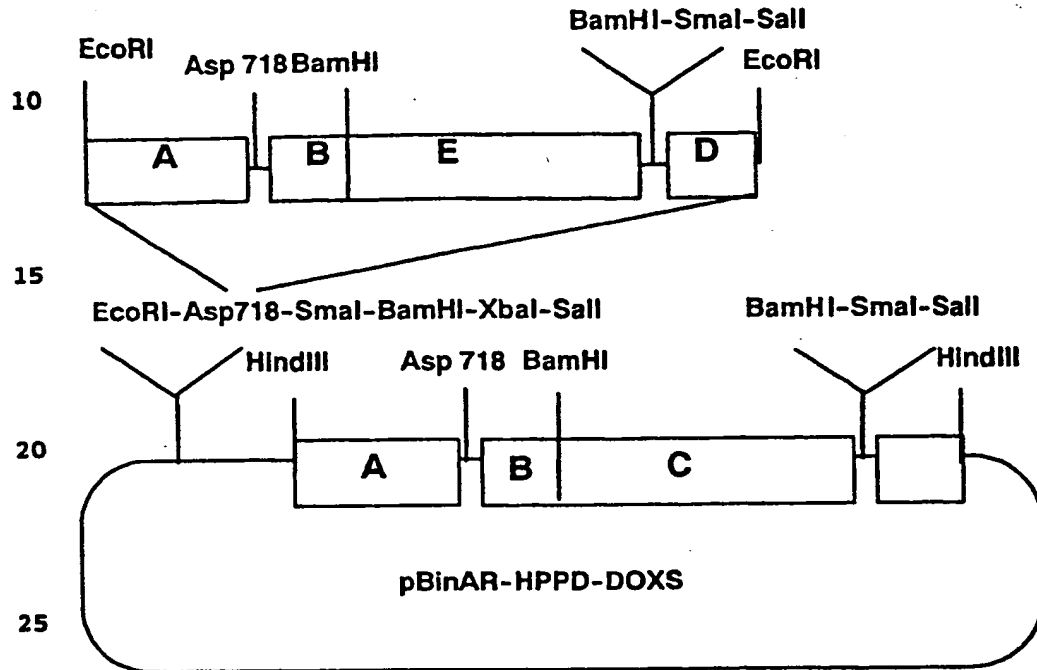
45

0817/00006

2

Figure 13

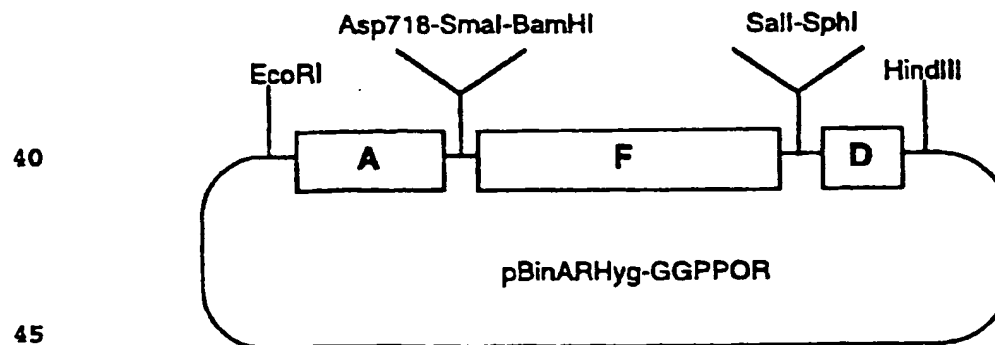
Binary vector for overexpression of the HPPD gene from *Streptomyces avermitilis* and the DOXS gene from *E. coli* in 5 plastids of transgenic plants.



30 Figure 14

Binary vector for overexpression of the GGPPOR gene from *Arabidopsis thaliana* in plastids of transgenic plants.

35

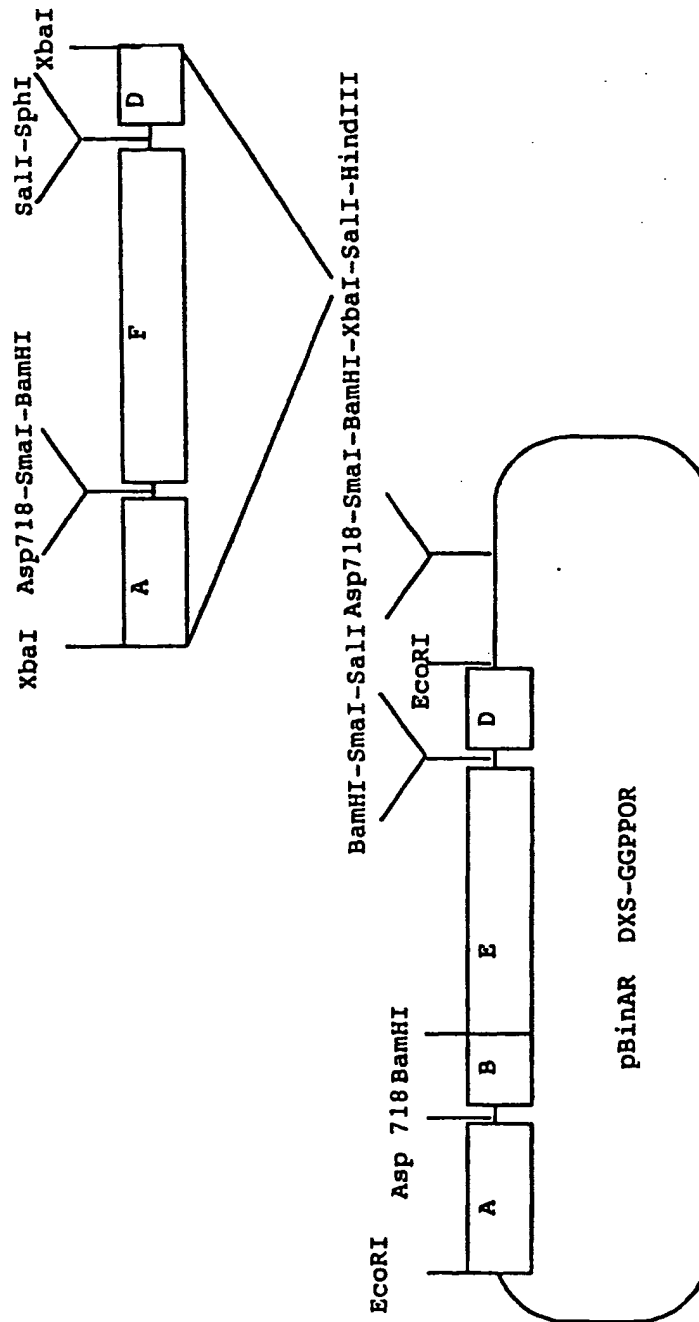


0817/00006

11a/11

Figure 15

Binary vector for overexpression of the GGPPOR gene from *Arabidopsis thaliana* and the DOXS gene from *E. coli* in plastids of transgenic plants

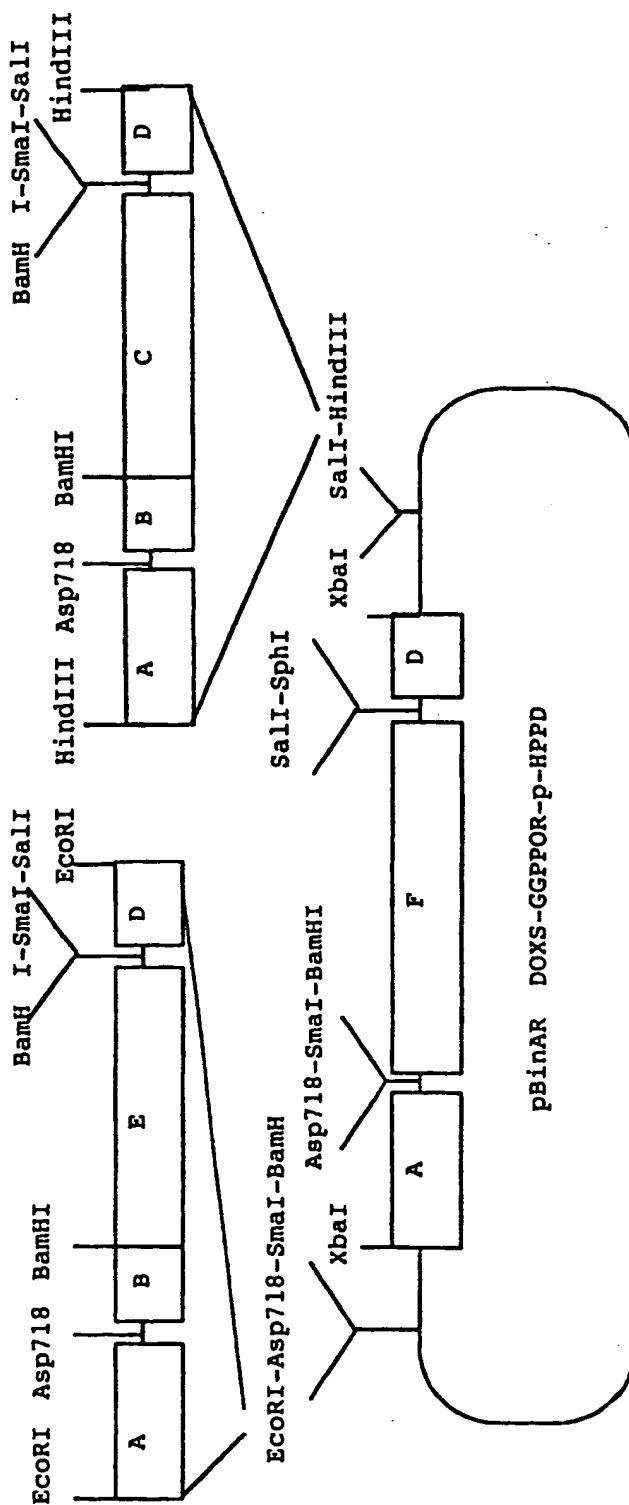


0817/00006

11b/11

Figure 16

Binary vector for overexpression of the DOXS gene from *E. coli*, the GGPPOR gene from *Arabidopsis thaliana* and the HPPD gene from *Streptomyces avermitilis* in the plastids of transgenic plants



**BINARY VECTOR FOR OVEREXPRESSING THE DOXS-GENE FROM E. COLI,  
THE GGPPOR GENE FROM ARABIDOPSIS THALIANA AND THE HPPD GENE  
FROM STREPTOMYCES AVERMITILIS IN THE PLASTIDS OF TRANSGENIC PLANTS**

